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Salivary Urea Nitrogen Dipstick as a Diagnostic Tool for Acute Kidney Injury

PhD student: Viviane Calice da Silva
Supervisor: Dr. Roberto Pecoits-Filho
Co-Supervisor: Dr. Peter Kotanko

Curitiba, Brazil.

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VIVIANE CALICE DA SILVA

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Supervisor: Dr. Roberto Pecoits-Filho
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ATA DA SESSÃO PÚBLICA DE EXAME DE TESE DO PROGRAMA DE PÓS-GRADUAÇÃO EM CIÊNCIAS DA SAÚDE EM NÍVEL DE DOUTORADO DA PONTIFÍCIA UNIVERSIDADE CATÓLICA DO PARANÁ.

Aos cinco dias do mês de maio de 2016, realizou-se a sessão pública de defesa de tese, **"SALIVARY UREA NITROGEN DIPSTICK AS A DIAGNOSTIC TOOL FOR ACUTE KIDNEY INJURY"** apresentado por **VIVIANE CALICE DA SILVA** para obtenção do título de Doutor; Área de Concentração: Medicina e áreas afins.

A Banca Examinadora foi composta pelos seguintes membros:

MEMBROS DA BANCA	ASSINATURA
Prof. Dr. Roberto Flavio Silva Pecoits-Filho (PUCPR)	
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De acordo com as normas regimentais a Banca Examinadora deliberou sobre os conceitos a serem distribuídos e que foram os seguintes:

Prof. Dr. Roberto Flavio Silva Pecoits-Filho (PUCPR) Conceito: Aprovada

Profª. Drª. Andrea Novais Moreno (PUCPR) Conceito: Aprovada

Prof. Dr. Marcelo Pilonetto (PUCPR) Conceito: APROVADO

Prof. Dr. Emannuel de Almeida Burdmann (USP) Conceito: APROVADO

Prof. Dr. Jochen G. Raimann (Renal Research Institute) Conceito: approved

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Observações da Banca Examinadora:

Prof. Dr. Roberto Flavio Silva Pecoits -Filho
Presidente da Banca Examinadora

Prof. Dr. Roberto Flavio Silva Pecoits -Filho
Coordenador do PPGCS PUCPR

Rua Imaculada Conceição, 1155 Prado Velho CEP 80215 901 Curitiba Paraná Brasil
Tel.: (41) 3271 2285 Fax: (41) 3271 1657 e-mail: ppgcs@pucpr.br www.pucpr.br

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“Do your little bit of good where you are; it’s those little bits of good put together that overwhelm the world.”

Desmond Tutu

Abbreviations list:

ADQI: Acute Dialysis Quality Initiative

AKI: Acute kidney injury

AKIN: Acute Kidney Injury Network

BIMC: Beth Israel Medical Center

BUN: Blood Urea Nitrogen

CKD: Chronic kidney disease

$CO(NH_2)^2$: urea

CUH: Cajuru University Hospital

GFR: Glomerular filtration rate

ESRD: End-Stage Renal Disease

H_2O: water

ICU: Intensive Care Unit

HRHDS: Hans Dieter Schmidt Regional Hospital

IGFBP7: insulin-like growth factor-binding protein 7

IL-18: Interleukin-18

ISN: International Society of Nephrology

KDIGO: Kidney Disease Improving Global Outcomes

KIM-1: Kidney injury molecule - 1

NGAL: Neutrophil gelatinase-associated lipocalin

NH_3: ammonia

NH_4^+: ion ammonium

OH^-: ion hydroxyl

QEH: Queen Elizabeth Hospital

RIFLE: Risk/Injury/Failure/Loss/End-stage

RRT: Renal replacement therapy

sCR: Serum creatinine

SUN: Salivary Urea Nitrogen

TIMP-2: tissue inhibitor of metalloproteinases-2

UO: urine output

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Abstract:

Introduction and Aims: Acute kidney injury (AKI) is very common worldwide, causing high morbidity and mortality, particularly in the developing world. Currently, simple, inexpensive and fast tools to help in the diagnosis and guide treatment are lacking. Salivary urea nitrogen (SUN) dipstick has been proposed as a bedside, screening method to detect AKI. The aim of this thesis was to evaluate the diagnostic performance of SUN to diagnose and monitor patients suffering from AKI in different settings around the world.

Methods: We measured SUN and blood urea nitrogen (BUN) in hospitalized patients diagnosed with AKI based on AKIN-criteria. Patients were evaluated in a cross sectional fashion (study 1) and were followed up for 7 days with daily (or every other day) (studies 2 and 3) measurements. After collection, saliva was transferred to a colorimetric SUN dipstick (Integrated Biomedical Technology, IN). The resultant test-pad color was compared to six standardized color fields indicating SUN of 5–14 (#1), 15–24 (#2), 25–34 (#3), 35–54 (#4), 55–74 (#5), and ≥75 (#6) mg/dl, respectively. BUN was determined by the urease method. AKI was stratified according to the AKIN and KDIGO classification. The diagnostic performance and agreement to severity of AKI were studied in all 3 population across studies using a standardized statistical approach: Bland-Altman analysis and linear mixed effects models to test agreement between SUN and BUN and receiver operating characteristics (ROC) statistics were used to test the diagnostic performance to diagnose AKI severity.

Results: Two hundred fourteen patients were enrolled in the 3 studies, 44 in the cross-sectional analysis and 170 in the follow up study (40 Brazil + USA and 130 Africa). SUN and BUN had a good agreement (Spearman rank Rs = 0.69; $p<0.001$). Diagnostic performance of SUN to diagnose AKI stage 3 was: AUC ROC 0.90 (95% CI 0.80-1.0) (Study 1) and AUC 0.81 (95% CI 0.63 to 0.98) (Study 2) and to diagnose AKI all stages AUC 0.82 (95% CI 0.78–0.87) (Study 3). These results were comparable to the BUN findings: AUC ROC 0.90 (95% CI 0.77-1.0) (Study 1), AUC 0.85 (95% CI 0.71 to 0.98) (Study 2) and AUC 0.82 (95% CI 0.59–1.0)(Study 3). BUN is underestimated by SUN consistently across studies, populations and days in the follow up, and discriminated with comparable accuracy.

Conclusions: According to the results presented in this thesis, SUN has an equivalent diagnostic performance compared to BUN to detect AKI, particularly in the most severe presentations. SUN reliably reflects BUN and follows its changes over time. Therefore, SUN testing associated with a thorough clinical evaluation may assist in the identification of patients with suspected AKI, especially under circumstances of limited health care resources and in the most remote settings.

Summary:

1- Introduction:

Kidneys are responsible for many important functions in the body such as excretory, endocrine and metabolic. Acute kidney injury (AKI) is a heterogeneous syndrome caused by multiple factors and diseases with important impact in morbidity and mortality to the affected patients. AKI is currently considered a global health problem due to its high incidence and prevalence worldwide, the elevated morbidity and mortality of patients and the high burden to healthcare systems [1]. As a consequence of international initiatives to increase awareness of the healthcare community to AKI, the reality of AKI demographic and geographic distribution and clinical presentation has been unveiled. Challenges to achieve the reduction of AKI morbidity and mortality are also being identified. Among these challenges, the need for simple, inexpensive and fast tools to help in the diagnosis and guide treatment of AKI has become apparent, particularly in low resource settings.

1.1 - Acute kidney injury definition, etiology and epidemiology:

AKI is defined as a syndrome caused by the abrupt loss of renal function, which is highly associated with increased patient's morbidity and mortality, as well as, increased risk of chronic kidney disease (CKD) in the long term [2]. AKI has three distinct main etiologies: a) pre-renal – a potentially reversible condition, in which the kidneys do not receive enough blood flow leading to their hypoperfusion, as occurs in cardiogenic, septic, hypovolemic shock and dehydration; b) renal – caused by injury to the renal cells, such as tubular necrosis (ischemic or toxic), interstitial nephritis, glomerular and vascular renal diseases, and c) post-renal - obstructive nephropathy due to nephrolithiasis or extrinsic compression [1].

According to the Kidney Disease Improving Global Outcomes Clinical Practice Guideline (KDIGO), AKI is defined as: a) increase in baseline serum creatinine by ≥0.3 mg/dl within 48 hours; or b) increase in serum creatinine to ≥1.5 times baseline, which is known or presumed to have occurred within the prior 7 days; or c) urine output < 0.5 ml/kg/h for 6 hours [1]. The variation in serum creatinine and urine output determines the AKI severity. Higher serum creatinine and lower urine output are common findings in the most severe degree of AKI; consequently, these patients present a higher probability of require renal replacement therapy (RRT). The presence of AKI during patient's hospitalization increases costs, time of hospitalization, mortality risk (**Figure 1**) and increases the risk of chronic kidney disease (CKD) development (**Figure 2**) in the long term in both hospital and community-acquired settings [3, 4]

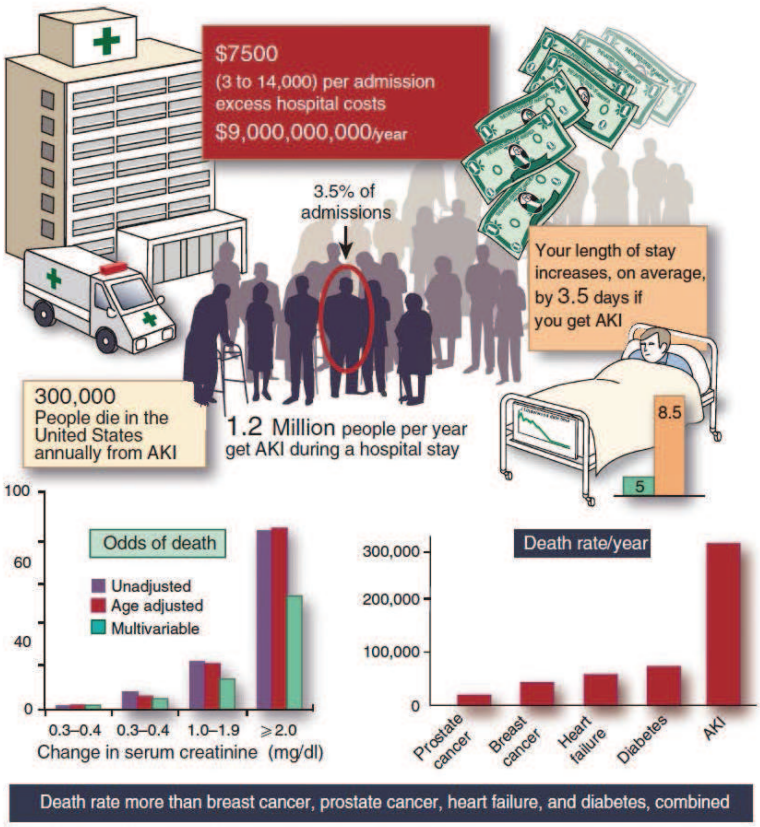

Figure 1: The global burden of AKI. Source: Lewington et al. Raising awareness of AKI, Kidney International, 2013, Sep; 84(3): 457-67.

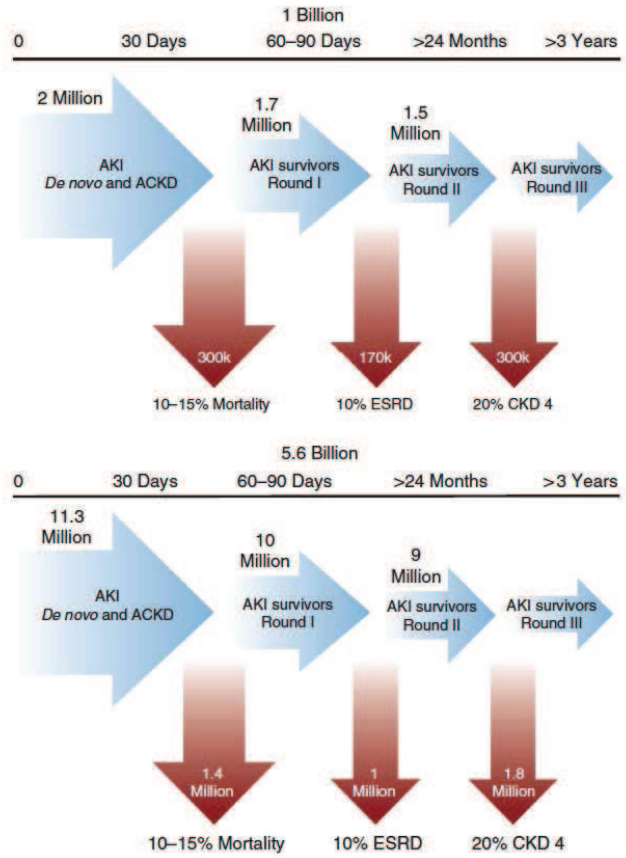

Figure 2: Estimated burden of AKI with progression to CKD and death across the world in a) high income countries and b) low income countries. Source: Lewington et al. Raising awareness of AKI, Kidney International, 2013, Sep; 84(3): 457-67.

AKI can occur in two different settings, namely hospital- and community-acquired. Hospital acquired AKI occurs mostly in developed countries, particularly in elderly patients and as a complication of pre-existing conditions, which leaded to the hospitalization. Community acquired AKI is more common in developing countries, where younger and previously healthy patients are more frequently affected with infectious diseases such as diarrhea, malaria, dengue, post-partum complications, leading to AKI [5].

Its estimated that AKI affects 3-13 million people per year and contributes to 1-7 million deaths every year [3]. In the developed world approximately 3.2-9.6% of hospital admissions are due to AKI, with overall in-hospital mortality of 20%, reaching 50% in the Intensive Care Unit (ICU) [6, 7]. AKI requiring RRT occurs in 6% of ICU patients, where a higher in-hospital mortality rate is observed (60%). Even after initial recovery of renal function and discharge from the hospital, AKI patients carry a higher risk of developing CKD in the following years, and also an adjusted long-term mortality risk of 1.4, which is proportional to the severity of AKI [7, 8].

In the developing world the incidence of AKI is underestimated due to the lack of population-level studies, especially in the lower and middle-income countries, where 85% of the world's population resides [3]. One of the most important projects developed to better understand the epidemiology and pattern of AKI worldwide was the AKI Global Snapshot, launched in 2014 by the International Society of Nephrology (ISN). This project is part of the 0 by 25, also organized by the ISN, an ambitious initiative with the aim of eliminating preventable deaths from AKI by the year of 2025, with a particular emphasis in low-income and middle-income countries [4]

An updated meta-analysis of studies utilizing the KDIGO or KDIGO-equivalent classifications to identify AKI was published in 2015. In this meta-analysis, data on AKI varied substantially across the studies (**Figure 3**). Approximately 5% of all patients required dialysis and AKI represented 20% of all hospital admissions, with an incidence corresponding to 3000-5000 per 1 million population per year. Another important finding was the fact that, in low-middle-income countries, the use of AKI classifications is increasing, allowing a better estimation of the real incidence of AKI in those areas. The

overall mortality of 21% observed in the pooled analysis is probably due to the predominance of mild stages of AKI. However, in patients with stage 3 or those who require dialysis presented a higher mortality (42% and 46%, unadjusted odds ratio 12.5 and 19.7, respectively) was identified[4]. Additionally to these observations, low-middle-income countries present an important lack of data of AKI in rural areas. Finally, it is clear that several studies produced in this area were single-center experiences, not available in the main medical journals.

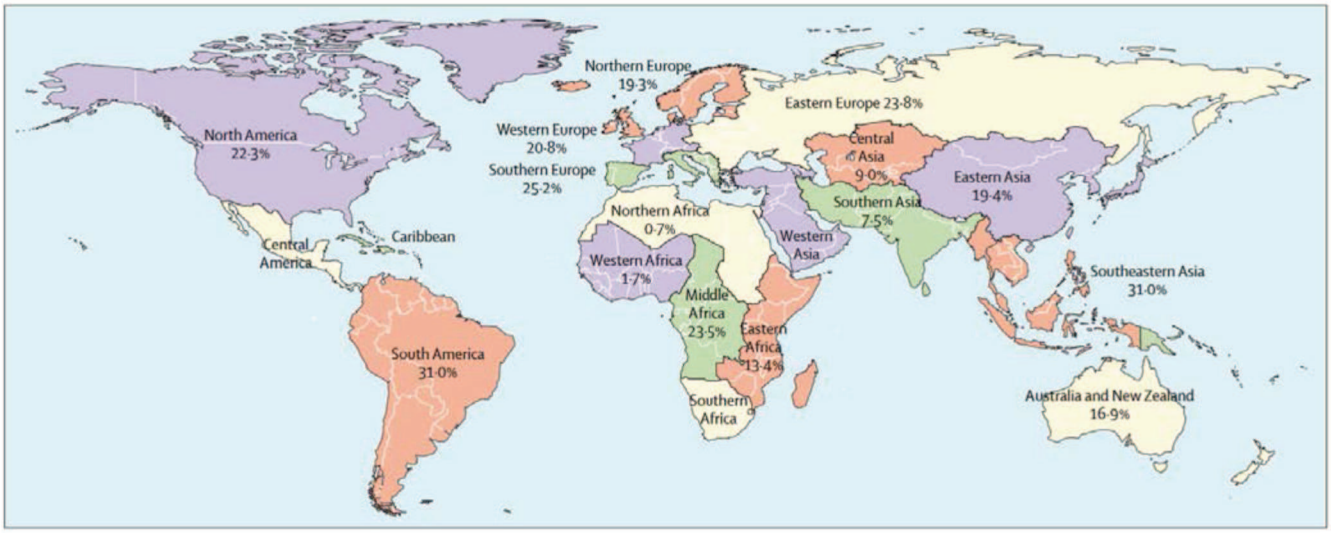

Figure 3: Pooled incidence of AKI worldwide shown per regions. Source: Mehta R. et al, www.thelancet.com. March 13, 2015.

Thus, the variability of risk factors distribution between countries and differences in the structure of healthcare facilities as well as accessibility of diagnostic tools, availability of dialysis units and treatment options are a main obstacle to the adequate management of AKI in those areas. These factors most likely have an impact in the identification of the magnitude and outcome of community-acquired AKI across the world [4].

ISN through the 0by25 initiative proposed an algorithm to raise awareness of AKI and reduce variation of the patients care called 5 Rs: risk assessment, recognition, response, renal support and rehabilitation (**Figure 4**). The intention is to establish criteria to be evaluated according the availability of resources in each area and guide health care workers decisions.

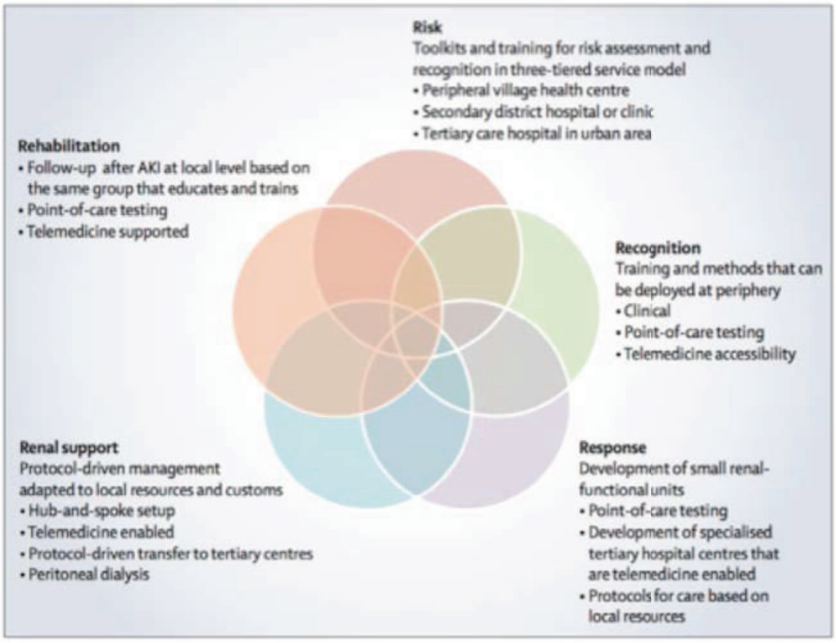

Figure 4: International Society of Nephrology 0 by 25 Initiative: Acute kidney Injury care based on the 5 Rs. Source: Mehta R. et al, The Lancet, March, 2015.

Recently, through the AKI Global snapshot, a cross-sectional study performed between September 29th and December 7th, 2014, AKI was assessed in 289 centers from 72 countries with different gross national incomes (GNI) recruiting more than four thousands patients suffering from this syndrome. In this study, AKI was identified to be 58% of times community-acquired AKI with 22% of patients needing renal replacement therapy (peritoneal dialysis performed only in low and low-middle income countries - LLMIC). Mortality rates were also identified and were similar in all countries regardless the GNI,

around 10%. The main causes of AKI were infectious disease, sepsis and dehydration in LLMIC and hypotension and shock in high-income countries. Renal recovery was more often in LLMICs irrespective of the need of dialysis[9].

1.2 - AKI diagnosis and classification:

Kidney function is currently evaluated through measurement of serum urea and creatinine and also through the determination of the glomerular filtration rate (GFR). GFR has been the best overall index of kidney function in health and disease [10, 11] and, can be measured or estimated. Measurements can be based on clearances of endogenous (creatinine, urea, cystatin C) or exogenous substances (inulin, iodexol, iothalamate) [12]. Recently, estimation equations were described and provide more reliable results [13-15]. One example of these equations widely used to eGRF is the Modification of Diet in Renal Disease (MDRD) study equation with standardized creatinine expressed as per below [16]:

$eGFR(mL/min/1.73m^2) = 175 \times SCr^{-1.154} \times age^{-0.203} \times 0.742$ [if female] $\times$ 1.210 [if black]

For many years serum creatinine (sCR) and blood urea nitrogen (BUN) have been used to evaluate renal function, both in chronic and acute illnesses. Even though considered late markers of AKI, which means that the damage to the kidneys are already installed when the levels of these two increased in the blood, they are still considered the standard methods to access the kidney function in AKI, due to the broad availability and lower costs.

Serum creatinine, known as the waste product of muscle catabolism, is primarily excreted by the kidneys, where it is freely filtered, secreted by tubular cells, and in addition eliminated via extrarenal (via intestinal post degradation by intestinal flora) [11]. Creatinine generation is also increased by creatine intake in meat or dietary supplements [17]. The

tubular and extrarenal creatinine excretion lead to an overestimation of renal function [11] . Despite of that, creatinine is currently the only biomarker recommended by guidelines to diagnose and classify the severity of AKI, associated or not with urine output [18].

In the AKI setting the severity of the disease is evaluated through the application of AKI classifications available in the literature, based on the variation of serum creatinine compared to the baseline and/or changes in urine volume over time [1, 19].

The Acute Dialysis Quality Initiative (ADQI) published the first AKI classification in 2004 based on a Risk/Injury/Failure/Loss/End-stage (RIFLE), stratification [19]. In 2007, a modified version of the RIFLE criteria was published by the Acute Kidney Injury Network (AKIN). Based on this classification, the three first categories of Risk, Injury, and Failure corresponded to AKIN stages 1, 2 and 3, respectively [19, 20]. In 2012, KDIGO proposed a classification which aggregates the previous definitions [1] (**Figure 5a and 5b and Table 1**).

a)

	RIFLE criteria		
		sCreatinine	Urine output criteria
Increasing severity	Risk	↑ sCrea × 1.5	< 0.5 ml/kg per h × 6 h
	Injury	↑ sCrea × 2	< 0.5 ml/kg per h × 12 h
	Failure	↑ sCrea × 3 or ⩾ 0.5 mg/dl if baseline sCrea ↑ > 4.0 mg/dl	< 0.3 ml/kg per h × 24 h or anuria × 12 h
Outcome	Loss	Complete loss of renal function > 4 weeks	
	End-stage	End-stage renal disease	

b)

	AKIN criteria		
		sCreatinine	Urine output criteria
Increasing severity	Stage 1	↑ sCrea × 1.5 or ↑ ⩾ 0.3 mg/dl in sCrea	< 0.5 ml/kg per h × 6 h
	Stage 2	↑ sCrea × 2	< 0.5 ml/kg per h × 12 h
	Stage 3	↑ sCrea × 3 or ↑ ⩾ 0.5 mg/dl if baseline sCrea > 4.0 mg/dl	< 0.3 ml/kg per h × 24 h or anuria × 12 h
	Patients who receive RRT are considered to have met stage 3 criteria, irrespective of the stage they are in at the time of RRT		

Figure 5: Stratification of AKI severity according: **a)** the RIFLE criteria; **b)** Acute Kidney Disease Network (AKIN) classification. Source: Kai Singbartl et al. Kidney International 81, 819-825, May 1, 2012

	Serum Creatinine Criteria AKIN	Serum Creatinine Criteria - KDIGO	Urine output Criteria AKIN and KDIGO
Stage 1	Increase in sCr of **0.3mg/dL or 150%-200% (1.5-2 fold)** baseline	Increase in sCr of **0.3mg/dL or 150%-200% (1.5-2 fold)** baseline	Urine output of **<0.5mL/kg/h** for **>6** hours
Stage 2	Increase in sCr of **200%-300% (2 – 3 fold)** from baseline	Increase in sCr of **200%-300% (2–3 fold)** from baseline	Urine output of **<0.5mL/kg/h** for **>12** hours
Stage 3	Increase in sCr of **> 300% (3 fold)** (Or sCr of more than **4.0mg/dL with an acute increase of >0.5mg/dL**, Or requiring **RRT**)	Increase in sCr of **> 300% (3 fold)** (Or sCr of more than **4.0mg/dL with an acute increase of >0.5mg/dL**, Or requiring **RRT**)	Urine output of **<0.3mL/kg/h** for **>24** hours Or **anuria for >12** hours

Table 1: Stratification of AKI severity according KDIGO Clinical Practice Guideline for Acute Kidney Injury showing the differences between AKIN and KDIGO classifications. Source: Mehta R. et al, www.thelancet.com Published online March 13, 2015.

1.2.1- Other biomarkers in AKI:

New biomarkers to assess kidney damage have been proposed in the last few years. These biomarkers started to be studied due to the need for earlier detection of kidney damage, which may facilitate earlier interventions within the narrow window of reversibility, to help to evaluate the effectiveness of the treatment applied, and also guide pharmaceutical development [21-25]. Some examples of these biomarkers are shown in **table 2**.

Biomarker	Biomarker type	Medium	Diagnostic performance (average AUC ROC) for AKI (as per various plasma creatinine-based definitions)

Cystatin C	Functional Markers	Plasma, urine	0.62 to 0.82 (plasma), 0.50 to 0.84 (urine)
Neutrophil Gelatinase-Associated Lipocalin (NGAL)	Up-regulated proteins	Plasma, urine	0.64 to 0.85 (plasma); 0.50 to 0.96 (urine)
Kidney Injury Molecule -1 (KIM-1)	Up-regulated proteins	Urine	0.55 to 0.78
Liver Fatty acid binding protein (L-FABP)	Up-regulated proteins	Urine	0.95
Interleukin-18 (IL-18)	Up-regulated proteins	Urine	0.55 to 0.73
Gammaglutanyl transpeptidase	Enzymes	Urine	0.62 to 0.95
Alkaline phosphatase	Enzymes	Urine	0.56 to 0.86
Alpha-glutathione s-transferase (α-GST)	Enzyme	Urine	0.58 to 0.89
Pi-glutathion s-transferase	Enzyme	Urine	0.54 to 0.93
TIMP-2 and IGFBP7	Up-regulated proteins	Urine	0.80

Table 2: Biomarkers of renal function with type of biomarker, where they can be measured and their diagnostic performance, respectively. Courtesy from Raimann J.G. - adapted from Edelstein, C. L. Adv Chronic Kidney Dis, 2008,15(3): 222-234 and Vijayan, A. Am J Kidney Dis, 2016, (16) S0272-6386.

All the biomarkers presented above are promising, but present at the moment, limitations to their immediate application in the clinical practice, such as elevated costs, the need for a laboratory structure and lack of validity outside of the subset of patients with ischemia injury (e.g. KIM-1, NGAL, and IL-18) [23, 26].

1.3 – Motivation for the development of bedside, simple and inexpensive diagnostic tools in AKI

The studies presented in this thesis were initiated as a result of a multicentre, multinational effort to improve the diagnostic capability of remote and/or disadvantages areas worldwide

to detect the occurrence of acute kidney injury. Health care resources such as medications, equipment, trained personal and particularly screening strategies are scarce in these areas. Moreover, renal replacement therapy is not available in many regions [27]. Another important barrier to AKI recognition and treatment is the limited awareness of AKI in terms of diagnosis, treatment, and management by health care workers [3, 28, 29]

Perhaps most importantly to the present studies is the barrier represented by the limitations of the current diagnostic tools to screen and monitoring kidney function in specific settings. The current recommendations for diagnostic tools in AKI are first based on urine output, difficult to monitor in small hospitals and healthcare centers in rural areas and in low resource settings not only due to the lack of protocols but also of available personnel and, second based on the use of serum creatinine, which is a laboratory-dependent method.

1.3.1 - Salivary Urea Nitrogen (SUN)

As mentioned above, urea nitrogen is a biomarker utilized to assess renal function. It is produced by the liver and is a waste product of protein digestion and cellular decomposition. It is freely filtered by the glomerulus and passively reabsorbed in the proximal and distal tubule and, therefore, underestimates glomerular filtration rate (GFR) due to these mechanisms of secretion and reabsorption. High-protein intake, volume depletion, and gastrointestinal bleeding can also cause an increase in blood urea nitrogen levels, which could lead to a false interpretation of kidney dysfunction [30]. An important characteristic of urea nitrogen is the fact it can be found in many others fluids, such as tears, blood, saliva and cerebrospinal fluid [31].

Salivary urea nitrogen (SUN) was for the first time described by Wright in 1841, in a case report of a patient who developed a uterine tumor, consequently ascites and a decline in urine output. He believed that once the patient could not eliminate the urea through the urine, her organism developed compensatory mechanisms to eliminate urea through ascites and the saliva avoiding the neural manifestations of this excretion product accumulation[32]. Urea reaches saliva by diffusion transports and, associated with bicarbonate, is responsible for the buffer capacity of this fluid [33-35].

Since 1840s, many studies were performed with the aim to prove the diagnostic capability of SUN to estimate renal function. In 1922 Hench and colleagues described a positive relationship between saliva and blood urea levels, especially in patients with urea accumulation in the circulation. The determination of urea for this study was performed by the urease method. Salivary stimulation was proposed as a treatment for urea retention when patients had a low urine output and could not eliminate BUN through urine anymore [36].

Later in 1923, Hench proposed a salivary urea nitrogen index and showed a distinct and continuous parallelism of the SUN and BUN values [37]. The salivary urea index, defined as the number of milliliters (mL) of 5% solution of mercuric chloride used for each 100 mL of saliva, increased directly with the degree of urea retention as indicated by BUN. The SUN index had a sensitivity of 91% for urea retention detection. He concluded that the SUN index could be a substitute for BUN determination [37].

Barnett et al in 1929 studied the influence of saliva stimulation in the SUN results. In this study samples were collected with and without stimulation, whole saliva and parotid saliva were used to perform the measurements. Stimulation was conducted using solutions

of tartaric acid (0.05 to 0.5%). SUN measured in non-stimulated saliva had a better agreement with BUN when measured in both whole saliva and parotid salivary samples [38].

Later, studies were conducted to analyze SUN in specific salivary glands such as the parotid [39]. Mean parotid urea concentrations were reported to range from 73 to 95% of serum levels. Forland et al in 1964 proposed that SUN measured in the saliva collected from parotid glands could be used to monitoring hemodialysis patients. The correlation coefficient of mean values for BUN and SUN monitored during hemodialysis was 0.99 despite rapid changes in BUN and SUN levels during treatment [40].

The first study using a test-strip method to measure SUN was done by Akai and his collaborators, in 1983 when a dipstick was created to assess the urea nitrogen levels in saliva. The urease-containing (urease-bromocresol-green method) test strip and an automatic reflectance spectrometer were used to conduct these analyses. A saliva/serum ratio of approximately 1.03 was found with this method [41, 42].

Some years later, 1987, Sein et al published another study where significant correlation coefficients (r=0.74 and r=0.99) between SUN and BUN in non-stimulated whole saliva were found in 56 healthy subjects and in 50 patients undergoing hemodialysis respectively[43].

Another study performed by Cardoso et al. in 2009 found a significant correlation between SUN and BUN ($r=0.91$, $p<0.001$) in both 78 healthy subjects and 154 CKD patients. The SUN had a sensitivity and specificity of 100% in both groups in this study. A serum enzymatic colorimetric assay (urea colour 2R, Wiener Laboratory, Argentina) was

adapted for the SUN measurements (Cardoso et al., 2009). Until this date, all the methods studied to evaluate SUN were laboratory dependent [37, 40, 44, 45].

In 2007 a dipstick method was developed by Integrated Biomedical Technology, IN, USA, which allows the assessment of SUN, without additional devices, at the bedside (**Figure 6**). For the employment of this method, 50 μL of liquid saliva are transferred to the test pad of the colorimetric SUN dipstick (Integrated Biomedical Technology, IN).

Figure 6: Salivary Urea Nitrogen dipstick bottle and legend.

The SUN is cleaved by urease present in the test pad into ammonia and hydroxyl ions, which change the pH and consequently the test-pad color. In 1 min the result can be read and the test-pad color compared to six standardized color fields indicating SUN concentrations (**Table 3**). In the urea cleavage-process urea in contact with water is cleaved by urease enzyme and becomes ammonia and carbon dioxide. Ammonia in contact with water becomes the ion ammonium and hydroxyl. The release of hydroxyl ions will alter the pH, which leads to changes in the test-pad color reflecting the salivary urea nitrogen result (**Table 4**).

SUN test pad number (SUN range, mg/dL)	Color of the test pad
1 (5-14)	

2 (15-24)	
3 (25-34)	
4 (35-54)	
5 (55-74)	
6 (≥ 75)	

Table 3: Saliva urea nitrogen test pad number and respective SUN ranges.

Salivary urea nitrogen cleavage reaction
Urease $CO(NH_2)^2 + H_2O \longrightarrow 2\ NH_3 + CO_2$ (Urea) $NH_3 + H_2O \longrightarrow NH_4^+ + OH^-$ pH Indicator + OH^- ⟶ pH Indicator (Yellow) (Green/Blue)

Table 4: Outline of the saliva urea nitrogen chemical reaction.

The SUN results are divided in six ranges from the lower to the higher levels and are represented by the colors and test-pad numbers showed above. Test-pad result 1 refers to SUN between 5 and 14 mg/dL, test-pad 2 (15–24 mg/dL), test-pad 3 (25–34 mg/dL), test-pad 4 (35–54 mg/dL), test-pad 5 (55–74 mg/dL), and test pad 6 (≥75 mg/dL). The greater the test-pad number the worse the kidney function (**Figure 7**).

Raimann et al published a study where 68 CKD patients in the stages 1 to 5D were studied. The elevated BUN was diagnosed using this SUN dipstick; non-stimulated whole saliva was collected to perform the measurements. SUN and BUN were correlated significantly (RS = 0.63; $p < 0.01$). Elevated BUN was diagnosed by SUN determination

with an AUC ROC curve of 0.90 (95 % CI 0.85–0.95) and an inter-observer coefficient of variation of 4.9 % at SUN levels >50 mg/dL and within-sample reproducibility of 90 %[46].

Some biological factors may alter the BUN and SUN relationship, in particular urease-producing oral bacterial flora, which may give rise to falsely low SUN values this, however, does not affect the agreement at higher levels of SUN[47]. Another limitation is the circadian rhythm that salivary glands have, potentially leading a different secretion rate of some saliva compounder according the time of the measurement in relation to the time of the day. This suggests that the agreement between SUN and BUN may differ at different times of the day [48, 49].

Based on these observations related to the potential use of SUN and a bedside, easy to use and quick method to detect and monitor kidney function in low resources areas, we decided to perform a series of studies, which generated this thesis.

2 - Objectives:

- To evaluate the diagnostic performance of salivary urea nitrogen (SUN) dipstick compared to the standard method, blood urea nitrogen (BUN), to confirm renal dysfunction in hospitalized patients suffering from acute kidney injury in a cross-sectional setting (addressed in the study 01).
- To analyze the capability of the SUN strips in detect changes in BUN over a period of time up to seven days according the treatment applied to AKI patients (addressed in the study 02).
- To identify the diagnostic performance of SUN strip in detect kidney dysfunction and also changes of BUN over time in an adult cohort of hospitalized patients with AKI in a low resource setting (addressed in the study 03).

3- Methods:

3.1 – Salivary urea nitrogen dipstick

Non-stimulated saliva was collected within 4 hours of blood collection for the determination of BUN and other biochemical parameters such as serum creatinine (measured in hospital labs and according standard techniques in each center). Subjects were asked to refrain from eating and drinking for at least 15 minutes and then to provide 1 to 2 mL of saliva. Saliva was collected in a plastic cup and allowed to separate in a liquid and foamy phase over a period between 1 to 3 minutes. Fifty µL of liquid saliva were transferred to the test pad of the colorimetric SUN dipstick (Integrated Biomedical Technology, IN). The color of the test pad was read after one minute and compared to 6 standardized color fields indicating SUN concentrations of 5–14 (color pad #1), 15–24 (#2), 25–34 (#3), 35–54 (#4), 55–74 (#5), and ≥75 (#6) mg/dL, respectively [23]. Trained nephrologists on site who were unaware of the BUN levels at the time of SUN collection performed the SUN measurements. Certified and experienced personnel using standard techniques measured blood chemistries in the hospital's laboratory.

3.2 – Blood analyses:

Blood chemistries (urea or BUN, creatinine) were measured in the hospital laboratory by certified and experienced personnel using standard techniques according each setting (Advia 1200, Siemens, Flexor Junior Clinical Chemistry Analyser [Vital Scientific, Dieren, The Netherlands] or a Mindray Chemistry Analyzer BS-120 [Shenzen Mindray Bio-Medical Electronics Company, ShenZen, China] and by VITROS 5,1 FS Chemistry System.

In Brazil and Africa were urea is measured in different units all the values were converted to BUN (mg/dL) according it is used in the US, also to allow correct comparison between BUN and SUN.

3.3 - Cohort selection and data extraction

- Inclusion criteria:
    - Hospitalized patients;
    - Older than 18 years old;
    - Diagnostic of renal disease including a) AKI or suspicious AKI during the hospitalization (worsening of creatinine or decrease of urine output according the criteria proposed by the AKI classification chosen for the study group (AKIN); b) Acute kidney disease/disorder no AKI (AKD) and, c) Chronic kidney disease (CKD) no stage 5D with worsening of renal function.
    - Able to spit to provide saliva samples;
    - Able to give the informed consent;
- Exclusion criteria:
    - CKD stage 5D
    - Not able to spit and provide enough saliva samples and;
    - Not able to provide the informed consent.
- Study phases:
    - 1) Cross-sectional study performed at Hans Dieter Schmidt Regional Hospital, Joinville/SC, Brazil (2013-2014).
    - 2) Follow-up study performed in 4 different centers:
        - Brazilian centers (2014-2015):

        - Hans Dieter Schmidt Regional Hospital (HRHDS), Joinville/SC
        - Cajuru University Hospital (CUH), Curitiba/PR
    - USA center (2011):
        - Beth Israel Medical Center (BIMC), New York NY.
    - Africa center (2015):
        - Queen Elizabeth Center Hospital, Blantyre, Malawi.

- Data extraction:

  Hospitalized patients were screened from the lab routine according creatinine levels. Those patients with serum creatinine greater than the laboratory reference values according the gender had the charts reviewed for the research group and if AKI was detected or worsening of previous stage of CKD, patients were approached to participate on the study and applied the informed consent. Demographic, clinical and laboratorial data were collected to all patients whom agreed to participate after their consent according each study phase.

3.4 – Covariates selection

- Collected once:
    - Demographic variables: age, gender, race, weight and height;
    - Baseline laboratorial variables: previous serum creatinine and blood urea nitrogen;
    - Additional variables: image results to help in the detection of previous renal disease and also in the etiology of AKI.
- Collected according the study phase:

- Cross-sectional study collected once:
  - Clinical variables: urine output when available;
  - Laboratorial variables: baseline and actual serum creatinine and blood urea nitrogen and saliva urea nitrogen.
- Follow-up study collected daily or each two days (Africa):
  - Clinical variables: urine output when available or referred for the patient and presence of thirst;
  - Laboratorial variables: serum creatinine and blood urea nitrogen and saliva urea nitrogen.

3.5 – Statistical Analyses

Data of studied subjects were stored in a database maintained by the principal investigators in each site. De-identified data were used for analysis in compliance with local and federal regulations. For the cross-sectional study the statistical analyses were performed in SPSS 20 (SPSS© IBM© Statistics). For the prospective study analyses were performed in R 3.2.1 (codename 'World-Famous Astronaut'; R Foundation for Statistical Computing; Vienna, Austria) additionally using the packages *plyr, sandwich*, *nlme*, *multcomp*, *pROC* and *ggplot2* [50].

Descriptive statistics included measures of central tendency (mean, median) and spread (standard deviation; quartiles, interquartile range). SUN ranges and BUN were correlated by Spearman's rank test. In the first study Kruskal-Wallis with pairwise comparison was employed to compare BUN at different SUN levels. The diagnostic performance of SUN and BUN, respectively, to discriminate AKIN 3 from other AKIN stages was assessed in terms of sensitivity and specificity, areas under the receiver

operating characteristic curve (AUC ROC), and predictive values of positive and negative tests. A test result was defined as true positive (TP) when SUN or BUN levels, respectively, were above a threshold value (Testpad #4 for SUN and 47 mg/dL for BUN) in the presence of AKIN 3, and as true negative (TN) if the SUN level was below the threshold in the absence of AKIN 3.

In the prospective studies the agreement between SUN and BUN was tested employing Analysis of Variance (ANOVA) with a post-hoc Bonferroni correction for multiple testing. Differences between BUN and SUN (transformed to a continuous variable by choosing the midpoint for each range) were displayed as error bars (with 95% confidence intervals) at different days and depicted as a modified Bland-Altman plot using BUN as the "gold-standard" [51, 52].

Agreement between SUN and BUN over the entire period was also tested by development of linear mixed effects models, which assumes different random intercepts for each subject and random slopes for each day-to-day period to account for variations between the days where measurements were conducted. Diagnostic performance of SUN and BUN to discriminate AKIN III versus earlier stages and also with all AKI stages combined were analyzed by predictive values (i.e. sensitivity and specificity after defining AKIN III as the binary outcome (study 1 and 2)), and the area under the receiver operating characteristics curve at each of the observation days [53]. Additionally, in the third study parallel analyses of sensitivity and specificity were done to evaluate the diagnostic performance of SUN combined to the clinical parameters collected: a) presence of thirst and, b) lower urine output (UO)[54]. Also, in this last study, cox proportional hazard models were constructed to evaluate the predictors of death in this population. Kaplan-Meier curves were used to

assess the mortality risk in patients stratified by the absence or presence of SUN levels >14 mg/dL (i.e. SUN > test pad #1). All surviving patients were censored at Day 15. A two-sided p-value less than 0.05 was considered as statistically significant.

4 – Results

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Saliva urea nitrogen dipstick – a novel bedside diagnostic tool for acute kidney injury

Viviane Calice-Silva[1,2], Marcos A. Vieira[1], Jochen G. Raimann[3], Mary Carter[3], John Callegari[3], Nathan W. Levin[3], Peter Kotanko[3], and Roberto Pecoits-Filho[2]

[1]Pró-rim Foundation, Joinville, [2]School of Medicine, Pontificia Universidade Católica do Paraná, Curitiba, Brazil, and [3]Renal Research Institute, New York, NY, USA

Key words
acute kidney injury – blood urea nitrogen – diagnostic tools – saliva dipstick – saliva urea nitrogen

Abstract. Background and aims: Measurement of saliva urea nitrogen (SUN) may be valuable in the screening of kidney failure. Here we evaluate the diagnostic performance of SUN dipsticks in patients with acute kidney injury (AKI). Material and methods: We measured SUN and blood urea nitrogen (BUN) in hospitalized patients diagnosed with AKI based on Acute Kidney Injury Network (AKIN)-criteria. After collection, saliva was transferred to a colorimetric SUN dipstick. We then compared the resultant test-pad color to six standardized color fields indicating SUN of 5 – 14 (#1), 15 – 24 (#2), 25 – 34 (#3), 35 – 54 (#4), 55 – 74 (#5), and ≥ 75 (#6) mg/dL, respectively. We assessed the performance of SUN and BUN to discriminate AKIN 3 from earlier stages by the area under receiver operating characteristic curves (AUC ROC). Results: We enrolled 44 patients (59.5 ± 18 years, 58% female; pre-renal AKI: 67%; renal 24%; post-renal 9%) in AKIN stages 1 (59%), 2 (16%), and 3 (25%). SUN and BUN levels were correlated (Spearman rank Rs = 0.69; $p < 0.001$, n = 44) with the highest correlation in AKIN 1 (Rs = 0.63, p = 0.001, n = 26). SUN allowed a significant discrimination of AKIN 3 from earlier stages (AUC ROC 0.91; 95% CI 0.80 – 1.0), which was comparable to the diagnostic performance of BUN (AUC ROC 0.90; 95% CI 0.78 – 1.0). Conclusions: SUN dipsticks allow the discrimination of AKIN 3 from earlier AKI stages. This low-technology approach may aid the screening of severe AKI in areas where laboratory resources are scarce.

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Correspondence to
Roberto Pecoits Filho,
MD, PhD, FASN, FACP
School of Medicine,
Pontificia Universidade
Católica do Paraná, Rua
Imaculada Conceição,
1155, Curitiba/PR, Brazil
r.pecoits@pucpr.br

Introduction

The incidence and etiology of acute kidney injury (AKI) differs among health care settings and particularly between developed and developing countries [1, 2, 3, 4, 5]. In developed countries elderly patients predominate and more than 20% of AKI cases develop in intensive care units [3].

In contrast, in developing countries, AKI follows a bimodal presentation. In large urban centers, the pattern of AKI is similar to that found in the developed world. Age and comorbidities are associated with the severity of AKI, the stage of which predicts subsequent mortality risk [3, 4]. In rural areas, AKI will particularly affect children, adolescents, and women of childbearing age and is caused by specific infections or pregnancy-related complications [3, 4, 5, 6, 7]. Comorbidities are in general rare in this population.

The diagnosis of AKI is based on the presence of oliguria and a concurrent rise of serum creatinine levels. Both the RIFLE (Risk, Injury, Failure, Loss of kidney function and End-stage renal disease) classification and Acute Kidney Injury Network (AKIN) classification use urine output and serum creatinine changes for AKI classification [1, 2].

Most AKI cases are diagnosed as AKIN 3, indicated by a rise in serum creatinine more than three times the baseline value or a serum creatinine > 4 mg/dL (with an acute increase of at least 0.5 mg/dL), and an urine output < 0.3 mL/kg/h over a period of 24 hours or anuria for 12 hours [8, 9, 10, 11, 12, 13, 14, 15, 16]. AKIN 3 is associated with increased likelihood of progression to end-stage kidney disease and mortality.

Given the dire clinical consequences of AKI and the limited access to health care in general and laboratory medicine in particular in large regions of the world, an accurate yet simple and non-invasive test for renal dysfunction could be an important adjunct to prompt diagnosis and treatment of AKI.

Blood urea nitrogen (BUN) and serum creatinine are frequently used markers of

kidney function and both of them rise concurrently in chronic kidney disease (CKD) and AKI patients [17]. Urea distributes in total body water and in exocrine secretions such as saliva, tears, and sweat. In both healthy subjects and CKD patients stages 1 to 5D levels of saliva urea nitrogen (SUN) and blood urea nitrogen (BUN) are correlated [17, 18, 19, 20, 21, 22].

Recently, we explored the performance of a colorimetric SUN dipstick to diagnose BUN levels greater than 25 mg/dL in CKD patients. In a study involving 68 patients in stages CKD 1 – 5D, we observed sensitivities between 0.77 and 0.85 and specificities of 0.85 – 0.88, respectively. Minimal training allowed health care workers to use the SUN dipstick with a between-observer variability of 4.9% [23].

In the current study, we extend our experience with the SUN dipstick to patients with AKI.

Subjects and methods

Patient selection

We conducted this cross-sectional study between February and May 2013 in AKI patients hospitalized (both ICU and non-ICU settings) in the Hans Dieter Schmidt Regional Hospital, Joinville, Santa Catarina, Brazil. AKI was diagnosed based on AKIN criteria [1]. AKI etiology was classified in the three major groups, namely pre-renal, renal, and post-renal. After routine medical examination we approached potential subjects and informed them about the goal of this study. All patients gave written informed consent. We excluded patients with pre-existing CKD, those unable to give informed consent, and patients who were unable to collect sufficient volume of saliva. For logistic and medical reasons, in some patients dialysis treatment was initiated before the SUN measurements. The study was approved by the institution's ethics committee and was conducted in accordance with the Declaration of Helsinki and good clinical practice.

Measurements

Unstimulated saliva was collected within 4 hours of blood collection for the determination of BUN and other biochemical parameters. Subjects were asked to refrain from eating and drinking for at least 15 minutes and then to provide 1 – 2 mL of saliva. Saliva was collected in a plastic cup and allowed to separate in a liquid and foamy phase over a period between 1 and 3 minutes. 50 µL of liquid saliva were transferred to the test pad of the colorimetric SUN dipstick (Integrated Biomedical Technology, Elkhart, IN, USA). The color of the test pad was read after 1 minute and compared to 6 standardized color fields indicating SUN concentrations of 5 – 14 (color pad #1), 15 – 24 (#2), 25 – 34 (#3), 35 – 54 (#4), 55 – 74 (#5), and ≥ 75 (#6) mg/dL, respectively [23]. All SUN measurements were performed by a single trained nephrologist on site (V.C.S.) who was unaware of the BUN levels at the time of SUN measurement. Blood chemistries were measured in the hospital laboratory by certified and experienced personnel using standard techniques.

Statistical analysis

Data of studied subjects were stored in a database maintained by the principal investigator (V.C.S.). De-identified data were used for analysis in compliance with local and federal regulations. Statistical analyses were performed in SPSS 20 (SPSS© IBM© Statistics).

Descriptive statistics included measures of central tendency (mean, median) and spread (standard deviation; quartiles, interquartile range). SUN ranges and BUN were correlated by Spearman's rank test. Kruskal-Wallis with pairwise comparison was employed to compare BUN at different SUN levels. The diagnostic performance of SUN and BUN, respectively, to discriminate AKIN 3 from other AKIN stages was assessed in terms of sensitivity and specificity, areas under the receiver operating characteristic curve (AUC ROC), and predictive values of positive and negative tests.

A test result was defined as true positive (TP) when SUN or BUN levels, respectively, were above a threshold value (testpad #4 for SUN and 47 mg/dL for BUN) in the presence of AKIN 3, and as true negative (TN) if the SUN level was below the threshold in the ab-

Table 1. Demographic data.

	AKIN 1	AKIN 2	AKIN 3
Gender (Female/Male)	9/17	2/5	8/3
Mean Age (± SD) (years)	64.9 (15.9)	62.8 (22.2)	46.5 (17.2)
AKI etiology	Pre-renal:19 Renal: 5 Post-renal:2	Pre-renal:5 Renal: 2 Post-renal:0	Pre-renal:6 Renal: 3 Post-renal:2
	26	7	11
Median SUN midpoint (25^{th}; 75^{th} IQR) (test pad*)	19.5 (9.5; 29.5) #2	19.5 (9.5; 29.5) #2	64.5 (44.5; 75) #4
Median admission BUN (mg/dL) (25^{th};75^{th} IQR)	25.5 (19.4; 42.6)	58.5 (40.2; 64.1)	33.2 (23.2; 78.6)
Median BUN (mg/dL) (25^{th}; 75^{th} IQR)	32 (25.2; 39.2)	43.4 (35.9; 64.8)	84.4 (62.3; 92.1)
Median admission creatinine (mg/dL) (25^{th}; 75^{th} IQR)	1.31 (1.12; 1.72)	3.25 (2.49; 3.9)	3.81 (1.15; 5.95)
Median creatinine (mg/dL) (25^{th}; 75^{th} IQR)	1.57 (1.42; 1.66)	2.36 (2.15; 2.78)	4.4 (4.08; 6.11)
Dialysis dependent-HD (%)	None	2 (28.6%)	3 (27.3%)
Spearman rank (p-value)	0.63 (p = 0.001)	0.075 (p = 0.88)	0.41 (p = 0.217)

SD = standard deviation; IQR = Interquartile range. *5 – 14 mg/dL = color pad (#1); 15 – 24 mg/dL (#2); 25 – 34 mg/dL (#3); 35 – 54 mg/dL (#4); 55 – 74 mg/dL (#5), and ≥ 75 mg/dL (#6).

Table 2. Relationship between SUN and BUN. Kruskal-Wallis with pairwise comparison was employed to compare BUN at different SUN ranges showed significant differences between lower and higher SUN ranges (i.e., category #1 vs. #5 and #6). A lack of significant differences between BUN and SUN concentrations at lower SUN ranges (i.e., test pads #1 and #2 vs. #3 and #4) was observed.

SUN test pad number (SUN range, mg/dL)	Color of the test pad	Tests (n)	BUN (mg/dL)	
			Median	1^{st} – 3^{rd} quartile
1 (5 – 14)		12	28.6	22.4 – 38.3
2 (15 – 24)		11	35.7	27.5 – 37.3
3 (25 – 34)		4	38.4	31.7 – 41.1
4 (35 – 54)		7	45.2	32.2 – 50.4
5 (55 – 74)		6	62.9	48.5 – 51.8
6 (≥ 75)		4	84.3	79.9 – 85.5

sence of AKIN 3. False positive (FP) and false negative (FN) results were defined accordingly. Sensitivity was computed as TP/(TP+FN), and specificity as TN/(TN+FP). Optimal diagnostic thresholds were determined based on the maximum Youden's index (Youden's index = sensitivity + specificity – 1). Sensitivities and specificities associated with maximal Youden's index were then used to calculate for pre-test likelihoods (p(D+)) between 0 and 1 the associated post-test likelihoods of AKIN 3 conditional to the presence of a positive (P(D+|T+)) or negative (P(D+|T-)) test, respectively, by applying Bayes' theorem:

$$p(D+|T+) = p(D+) \times \text{sensitivity} / [p(D+) \times \text{sensitivity} + (1\text{-}pD+) \times FP]$$

$$p(D+|T-) = p(D+) \times FN / [p(D+) \times FN + (1\text{-}p(D+)) \times \text{specificity}]$$

In additional analyses we performed subset analyses a) to evaluate the correlation between BUN and SUN at different stages and etiologies of AKI and b) to compare the findings with and without those patients who received hemodialysis prior to the measurements. We also investigated the diagnostic performance of the SUN dipstick to diagnose BUN levels greater than 25 mg/dL to compare the diagnostic performance of the SUN test in AKI patients to sensitivity, specificity, and AUC ROC previously reported for CKD patients [23].

Results

We enrolled 44 patients (age 59 ± 18 years, 58% females) (Table 1). Causes of AKI were pre-renal (67%), renal (24%), and post-renal (9%). 59% of subjects were classified as AKIN stage 1, 16% as stage 2, and 25% as stage 3. Five patients (11.4%) were dialyzed prior to SUN measurements, 4 patients (9.1%) started dialysis after SUN measurements.

Comparative analysis

Kruskal-Wallis with pairwise comparison was employed to compare BUN at different SUN ranges showed significant differences between lower and higher SUN ranges (i.e., test pad #1 vs. #5 and #6). A lack of significant differences between BUN and SUN concentrations at lower SUN ranges (i.e., test pads #1 and #2 vs. #3 and #4) was observed (Table 2).

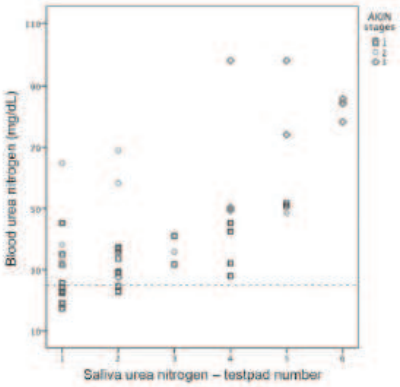

Figure 1. Relationship between SUN and BUN in 44 patients with AKI (Rs = 0.69, p < 0.001). The dotted line shows the upper limit of normal BUN (25 mg/dL), the symbols indicate AKIN stages: stage 1: square, stage 2: circle, stage 3: diamond. Pad #1: 5 – 14 mg/dL #2: 15 – 24 mg/dL #3: 25 – 34 mg/dL #4:35 – 54 mg/dL #5:55 – 74 mg/dL #6: ≥ 75 mg/dL.

Table 3. Correlation coefficient between SUN and BUN by AKI etiology.

	Pre-renal	Renal	Post-renal
N° patients	30	10	4
BUN median (25^{th}; 75^{th} IQR)	39.7 (33.4; 48)	29.6 (24.3; 48.5)	40.8 (25.7; 73.9)
SUN median (25^{th}; 75^{th} IQR)	2 (1.5; 3.5)	1.5 (1; 5)	4 (2.5; 4)
Spearman rank (p-value)	0.17 (0.5)	0.88 (0.001)	0.78 (0.225)

Correlational analysis

SUN and BUN levels were significantly correlated (Spearman rank Rs = 0.69; p < 0.001), with the highest correlation in AKIN 1 (Rs = 0.63, p = 0.001) (Figure 1) (Table 1). The correlations between SUN test pad and BUN differed by etiology of AKI, with highest correlation seen in renal AKI (Rs = 0.88), followed by post-renal (Rs = 0.78), and pre-renal (Rs = 0.17) (Table 3). A subset analysis excluding subjects who received dialysis prior to SUN and BUN measurements corroborated the correlation between SUN and BUN (Rs = 0.65, p < 0.001). In the 5 patients who received hemodialysis (HD) prior to study entry the median BUN (35.9 mg/dL) and SUN (test pad #2) were comparable to those seen in 39 patients who were not dialyzed (data not shown).

Diagnostic performance

Receiver operating characteristics curve analysis (ROC)

The diagnostic performance to discriminate AKIN 3 from earlier AKI stages was comparable between SUN (AUC ROC: 0.91 (95% CI 0.80 – 1.0)) and BUN (AUC ROC: 0.90 (95% CI 0.78 – 1.0)) as the diagnostic biomarker (Figure 2a, b). Subset analysis excluding subjects who received dialysis prior to SUN and BUN measurements resulted in an AUC ROC 0.88 (95% CI 0.75 – 1.0) and 0.87 (95% CI 0.7 – 1.0) respectively (Supplemental Figure 1 and 2).

Predictive values of SUN and BUN

Analysis of Youden's index indicated optimal thresholds to diagnose AKI stage 3 of 47 mg/dL for BUN and 35 – 54 mg/dL (test pad #4) for SUN (Figure 2b). These results were materially identical when we excluded patients in whom SUN was measured after dialysis initiation. In total there were 7 false positive (16%) and 1 false negative (2.3%) results when AKIN 3 was diagnosed based on the optimal diagnostic threshold test pad #4, resulting in a sensitivity of 0.91 (95% CI 0.58 – 0.99) and specificity of 0.79 (95% CI 0.61 – 0.91) (Tables 4 and 5). We found a positive predictive value of 0.59, negative predictive value of 0.97, a positive likelihood ratio of 4.3 (95% CI 2.16 – 8.5), and a negative likelihood ratio of 0.12 (95% CI 0.02 – 0.75).

Bayesian analysis

Employing Bayes' Theorem [26] and based on these test characteristics we calculated the post-test likelihoods for the presence of AKIN stage 3 as a function of pre-test likelihoods between 0 and 1. Notably, the diagnostic performance of SUN (Figure 3a) and BUN using the threshold found in this group (≥ 47 mg/dL) (Figure 3b) are comparable over a wide range of pre-test likelihoods.

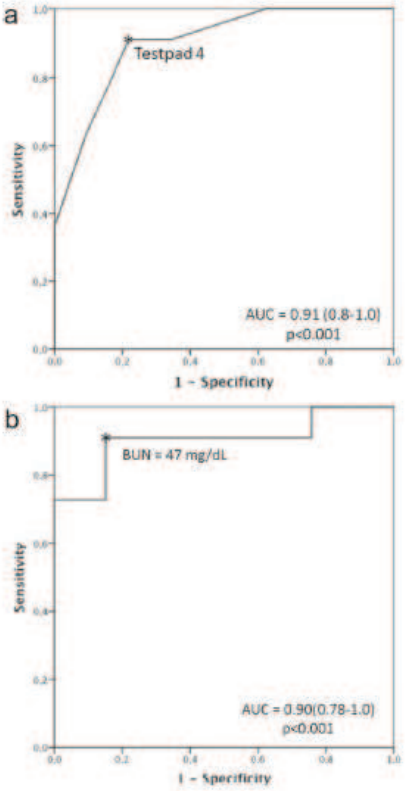

Figure 2. a: Diagnostic performance of SUN dipstick to diagnose AKIN stage 3; b: Diagnostic performance of BUN to diagnose AKIN stage 3.

Table 4. Relationship between SUN levels and presence or absence of AKI at AKIN stage 3. The data represent number of patients.

SUN test-pad	AKIN 1 or 2	AKIN 3	Total
#1	12	0	12
#2	10	1	11
#3	4	0	4
#4	4	3	7
#5	3	3	6
#6	0	4	4
Total	33	11	44

Sensitivity analyses

Since AKIN stage 2 is also associated with poor outcomes, we repeated the analysis by combining AKIN 2 + AKIN 3 to evaluate the diagnostic performance of SUN and BUN in this patient group. SUN discriminated AKIN 1 (n = 26) from AKIN 2+3 (n = 18) with an AUC ROC of 0.73 (95% CI 0.57 – 0.89). The optimal decision threshold was test pad #4 with a sensitivity of 0.61 and a specificity of 0.77. Of note, 6 out of 7 AKIN 2 cases and 1 out of 11 AKIN 3 cases were missed. The BUN AUC ROC was 0.89 (95% CI 0.79 – 0.99); the optimal BUN threshold was 47 mg/dL with a sensitivity of 0.78, and a specificity of 0.92.

In additional analyses, we investigated the diagnostic performance of the SUN dipstick to diagnose elevated BUN levels (defined as BUN > 25 mg/dL). We found a sensitivity of 0.88 (95% CI 0.78 – 0.99), specificity of 0.78 (95% CI 0.48 – 1.08), and AUC ROC of 0.84 (95% CI 0.71 – 0.98) (Supplemental Figure 3).

Discussion

Statement of principal findings

Our study indicates that SUN measured non-invasively by dipstick performs comparably well to diagnose AKI stage AKIN 3 when compared to BUN measured in a clinical laboratory.

In the comparative analysis it was possible to discriminate between high and low BUN levels using the SUN dipstick (i.e., test pad #1 versus test pads #5 and #6). SUN permitted the discrimination between high and low BUN subset analyses of a) patients who did not receive HD prior to the measurements and b) in patients with BUN concentrations above 25 mg/dL. While SUN test pad readings and BUN were correlated in the population as a whole, the best correlation was observed in AKIN stage 1. ROC curve analysis indicated comparably good diagnostic performances of SUN and BUN to discriminate AKIN stage 3 from less severe stages (Figure 2a, b) (Table 5). These findings are of potential relevance for patients with AKI in regions of the world with limited access to laboratory resources, particularly for patients at stage AKIN 3 who need to be treated more aggressively and require more resources for their treatment, including, dialysis.

Comparison to other studies

The fundamental paradigm underlying this diagnostic approach is that levels of SUN

Table 5. Sensitivity, specificity, positive and negative predictive value of the 6 SUN test pads to discriminate AKIN 3 from AKIN 1+2.

Test pad	AKIN classes	Sensitivity	Specificity	PPV	NPV
1	3	1.0	0	0.33	0
	1+2	0.75	0	1.0	0
2	3	1.0	0.36	0.33	1
	1+2	0.64	0	0.64	0
3	3	0.91	0.67	0.48	0.96
	1+2	0.33	0.09	0.52	0.04
4	3	0.91	0.79	0.59	0.97
	1+2	0.21	0.09	0.41	0.04
5	3	0.64	0.91	0.7	0.88
	1+2	0.09	0.36	0.3	0.12
6	3	0.36	1.0	1.0	0.83
	1+2	0	0.64	0	0.18

and BUN correlate as previously suggested. Akai et al. [19] studied SUN in 44 patients suffering from CKD and 12 control groups with a chromatographic method using a dry test strip and a handheld device measuring the reflectivity of the test strip after sample application. Sein et al. [20] found a significant correlation ($r = 0.74$, $p < 0.05$) between SUN and BUN in 56 healthy volunteers. In addition, a positive correlation ($r = 0.99$) between SUN and BUN collected during HD was previously described by Forland et al. [21]. Despite these encouraging results, biological factors may alter the relationship between SUN and BUN, in particular urease-producing oral bacterial flora which may give rise to spuriously low SUN [24].

In a study of 68 CKD patients stages 1 – 5D the presence of a BUN greater than 25 mg/dL was diagnosed by SUN dipstick with a sensitivity of 0.77, a specificity of 0.85, and AUC ROC of 0.85 [23]. These results are comparable to our current findings in AKI patients (AUC ROC of 0.91). In the current study in AKI patients, the SUN dipstick test had a sensitivity of 0.91 and specificity of 0.79 to diagnose AKIN 3, making it a useful tool to screen patients with suspected AKI in areas with limited resources. It is important to make sure that patients with severe AKI (at stage 3) are not misclassified as mild AKI, even at the expense of overcalling some other patients. In other words, the screening test should have a high sensitivity and high predictive value of a negative test. In that sense, SUN would serve as a screening, rather than diagnostic tool. Patients with AKI and a positive SUN test would need further and swift evaluation and confirmatory testing and/or intervention. In this study, the sensitivity and negative predictive value of the SUN test pad #4 were 0.61 and 0.74 to discriminate AKIN 2 + 3 from AKIN 1 and 0.91 and 0.97, respectively, to discriminate AKIN 3 from AKIN 1 + 2 (Table 5), indicating favorable screening characteristics in these groups of patients.

Based on these test characteristics in conjunction with a known or estimated pre-test likelihood of AKI, the post-test odds for the presence or absence of AKI at stage AKIN 3 can be easily calculated using Bayes' theorem [25]. For example, in a patient with a pre-test likelihood of AKI stage AKIN 3 of 0.20, a positive test ((defined as a SUN greater or equal to the value reflected by #4 test pad (as determined by our analysis using Youden's index)) would more than double the chances for having AKI to 0.52. However, if the same patient would have a negative SUN result (as defined by a SUN lower than the value reflected by #4 test pad) the probability of AKI would be 0.03 (Figure 3a, b).

Strengths of the current study

Generally, the concordance of the SUN dipstick test characteristics in distinct renal patient populations (the one in CKD patients in Europe and North America and the current study) carried out by different observers in different clinical settings and countries corroborates the usefulness of this test and generalizability of the current findings. The subset analyses at different AKIN stages and in those requiring HD and those not, suggests a general applicability of the test in various clinical settings especially in those patients in the worst disease severity. To further corroborate the previous findings we analyzed the diagnostic performance of SUN in those patients with BUN > 25 mg/dL (Supplemental Figure 3). Subset analyses showed a consistent correlation between BUN and SUN.

Limitations of the current study

The major limitation of the study is its small sample size and future studies in a larger

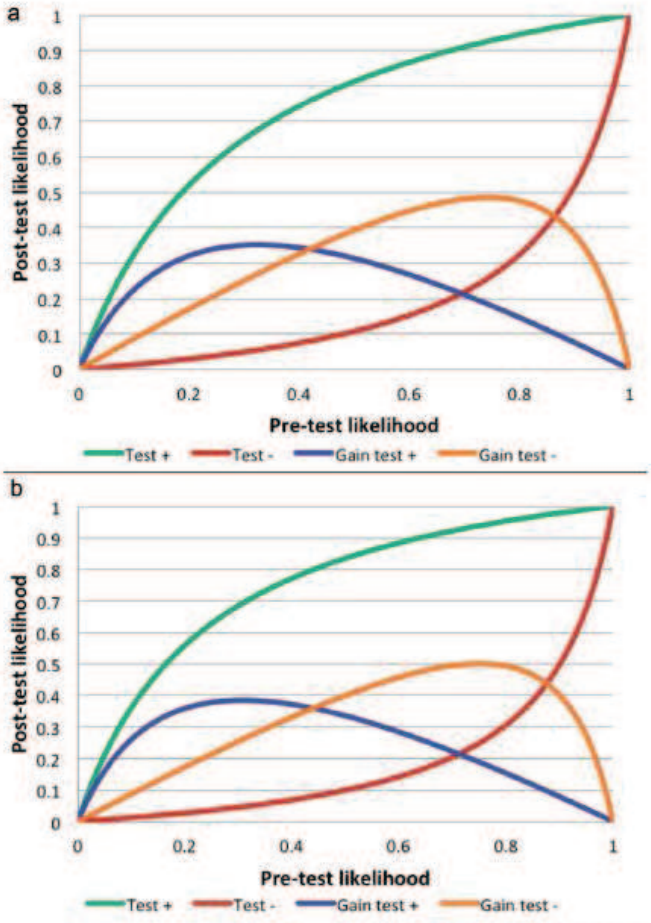

Figure 3. a: Relationship between pre- and post-test likelihood of AKIN 3 given SUN test sensitivity of 0.91 and specificity of 0.79; b: Relationship between pre- and post-test likelihood of AKIN 3 (BUN ≥ 47 mg/dL) given a BUN test sensitivity of 0.90 and specificity of 0.82.

and more diverse group of patients (including children, pregnant women, different races, AKI due to intoxications, and others) are necessary to make firm statements about questions such as diagnostic thresholds. In addition, our study did not address questions such as between-observer and within-observer variability. Other limitations relate to the fact that SUN test were not done in duplicate and non-AKI patients were not included in the study cohort.

Factors such as volume depletion, high protein diet, catabolic states, liver disease, and medication side effects can affect BUN levels independent of kidney function, rendering it a poor marker [26]. We did not have documentation of clinical conditions associated with high urea production, such as gastrointestinal bleeding, steroid use, and total parenteral nutrition with high nitrogen content. The correlation between SUN and BUN was poorest in patients with pre-renal AKI. While the cause for this finding is unclear, we speculate that patient's volume status may play a role. Patients with pre-renal are frequently volume depleted, which may result in decreased saliva flow rate, increased saliva transit time, and hence a higher level of urea degradation.

While being aware of these limitations we believe that SUN measurements in concert with medical history, clinical signs and symptoms, can assist early diagnosis and triage of AKI patients particularly in medically isolated areas. Of note, in the current study only one patient with AKIN 3 was not diagnosed correctly using the SUN dipstick.

Unanswered questions and future research

Further research will need to address means to improve concordance in the lower BUN ranges and to limit the effects of factors potentially confounding SUN levels. From a practical point of view, the technique of collecting saliva also needs consideration. Patients suffering from severe dehydration may not be able to produce sufficient saliva for the SUN measurement, and may require stimulation of saliva flow. While some subjects in this study had great difficulty to provide 1 mL of saliva, we were able to overcome this issue without the need for external stimulation of salivary flow by using a smaller saliva volume for the measurements. In some cases, the use of flow stimulants (such as chewing solid paraffin, sugared gum, pilocarpine hydrochloride, and others) may be considered. However, the inverse relationship between saliva flow rate (increased by stimulation) and SUN concentration may unfavorably alter the relationship between SUN and BUN [27, 28].

Meaning of the study

Semi-quantitative SUN measurements by a simple dipstick method is a low-tech,

easy to use, noninvasive, reliable, and cheap screening tool to identify patients with elevated BUN. SUN testing in tandem with a thorough clinical evaluation may assist in the evaluation of patients with suspected AKI, especially under circumstances of limited health care resources. Future studies in more diverse and larger populations, including children and pregnant women, are required to further explore the applicability of this test, which is currently being improved.

Acknowledgments

We are grateful to the laboratory team, our nephrology fellows and the clinical residents for their recruitment support, and to Research Ethic Committee of Hans Dieter Schmidt Regional Hospital. This work was in part submitted for presentation at the Renal Week 2013 of the American Society of Nephrology. During studies, V.C.S. was participating in an ISN Fellowship. Saliva dipsticks for detection of urea nitrogen levels were provided free of charge by the manufacturer (Integrated Biomedical Technology, Elkhart, IN, USA).

Conflict of interest

Dr. Calice-Silva was a Fellow of the International Society of Nephrology (ISN) and received funding from the ISN for a Research Fellowship at the Research Division of Renal Research Institute in New York City during this manuscript elaboration. Drs. Callegari, Carter, Kotanko, and Levin hold stock in Fresenius Medical Care. During this study, Dr. Pecoits-Filho was a recipient of a Scholarship from CNPq. All other authors have no financial interests to declare.

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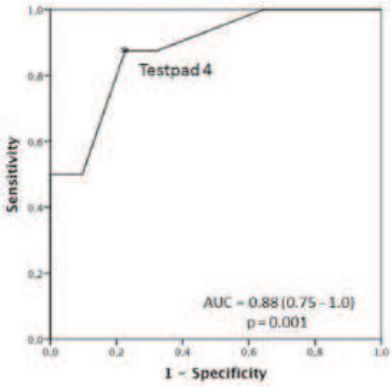
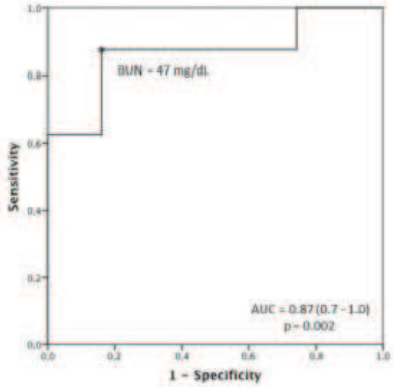
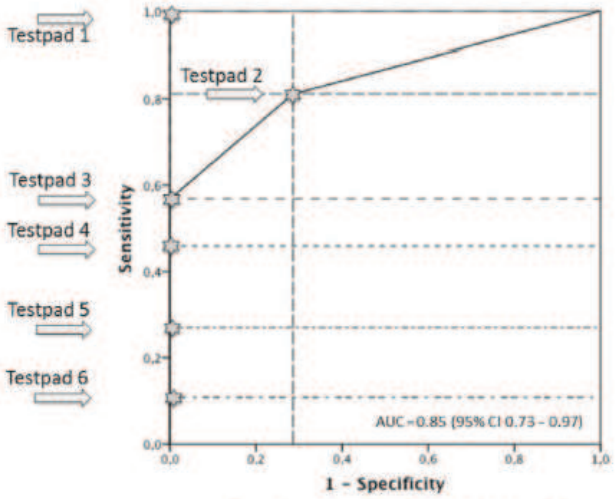
Supplemental figures



Supplement Figure 1. Subset analysis – diagnostic performance of saliva urea nitrogen dipstick to diagnose AKIN 3. Patients who received dialysis prior to SUN measurements were excluded in that subset analysis.



Supplement Figure 2. Subset analysis – diagnostic performance of blood urea nitrogen to diagnose AKIN 3. Patients who received dialysis prior to SUN measurements were excluded in that subset analysis.



Supplement Figure 3. Subset analysis – diagnostic performance of SUN to identify patients with a BUN level of greater 25 mg/dL.

4.2. Study 2: Saliva Urea Nitrogen continuously reflects Blood Urea Nitrogen after Acute Kidney Injury diagnosis and management: longitudinal observational data from a collaborative, international, prospective, multicenter study. Accepted: February 22, 2016, at Blood Purif DOI: 10.1159/000445041.

Original Paper

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Saliva Urea Nitrogen Continuously Reflects Blood Urea Nitrogen after Acute Kidney Injury Diagnosis and Management: Longitudinal Observational Data from a Collaborative, International, Prospective, Multicenter Study

Jochen G. Raimann[a] Viviane Calice-Silva[d, e] Stephan Thijssen[a] Fabiana Baggio Nerbass[d, e] Marcos A. Vieira[d] Pascal Dabel[b] Rhys Evans[f] John Callegari[a] Mary Carter[a] Nathan W. Levin[a, c] James F. Winchester[b, c] Peter Kotanko[a, c] Roberto Pecoits-Filho[d]

[a]Research Division, Renal Research Institute, [b]Beth Israel Medical Center New York, and [c]Icahn School of Medicine at Mount Sinai Health System, New York, USA; [d]School of Medicine, Pontificia Universidade Católica do Paraná, Curitiba, and [e]Pró-rim Foundation, Joinville, Brazil; [f]UCL Centre for Nephrology, Royal Free Hospital, London, UK

Key Words
Diagnostic tools · Acute kidney injury · Blood urea nitrogen · Saliva urea nitrogen · Saliva dipstick · Triage · Emerging countries · Healthcare · Dialysis

Abstract
Background: Acute kidney injury (AKI) is a growing global concern and often reversible. Saliva urea nitrogen (SUN) measured by a dipstick may allow rapid diagnosis. We studied longitudinal agreement between SUN and blood urea nitrogen (BUN) and the diagnostic performance of both. ***Methods:*** Agreement between SUN and BUN and diagnostic performance to diagnose AKI severity in AKI patients in the United States and Brazil were studied. Bland–Altman analysis and linear mixed effects models were employed to test the agreement between SUN and BUN. Receiver operating characteristics statistics were used to test the diagnostic performance to diagnose AKI severity. ***Results:*** We found an underestimation of BUN by SUN, decreasing with increasing BUN levels in 37 studied patients, consistent on all observation days. The diagnostic performance of SUN (AUC 0.81, 95% CI 0.63–0.98) was comparable to BUN (AUC 0.85, 95% CI 0.71–0.98). ***Conclusion:*** SUN reflects BUN especially in severe AKI. It also allows monitoring treatment responses. Video Journal Club 'Cappuccino with Claudio Ronco' at http://www.karger.com/?doi=445041.

Introduction

The global '0by25' initiative recently launched by the International Society of Nephrology (ISN) aims to eradicate preventable deaths resulting from acute kidney injury (AKI) [1]. Along with an increased community attention, AKI is being increasingly recognized as a chal-

J.G.R. and V.C.-S. contributed equally to this work.

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Jochen G. Raimann, MD, PhD
Research Division, Renal Research Institute
315 East 62nd Street, 4th Floor
New York, NY 10065 (USA)
E-Mail Jochen.Raimann@rriny.com

lenge to healthcare, particularly in regions with limited medical infrastructure [1, 2]. While AKI in developing countries is generally found in an older and sickly population, often hospitalized or in an intensive care unit, in developing countries AKI is more often community-acquired, and caused by potentially avoidable and modifiable conditions such as diarrheal diseases, malaria or obstetric complications [1, 2]. Given the nature of these conditions, timely management increases the probability of complete recovery. Rapid counteracting measures such as oral (or alternatively intravenous) rehydration or temporary renal replacement therapy may be key to restoring failed renal function. This emphasizes the need to develop tools that enable diagnosis to be made with minimal delay.

While clinical presentation and medical history are important indicators of the underlying condition, AKI-specific symptoms may be concealed, often within a myriad of nonspecific symptoms related to the underlying disease. Technological advances in the last decade have resulted in point-of-care testing (POCT) devices, which allow rapid assessments of not only renal parameters but also other factors ranging from metabolic and hormonal activities, to even specific cardiac parameters such as troponins and brain natriuretic peptides [3, 4]. These devices are generally costly and require training, calibration and regular comparison to laboratory assessments. Given the low level of available resources in healthcare settings in developing countries, a great need exists for low-cost and simple techniques, which are easy to use and which reliably indicate rapid changes in the renal function.

We have conducted the current study and analyses as an advancement to our earlier studies, in which we have shown that saliva urea nitrogen (SUN) measured by a simple dipstick method reflects elevated blood urea nitrogen (BUN) in a cohort of patients with chronic kidney disease [5], and permits the determination of AKI disease severity at hospital admission [6]. In this study, we have carried the analyses another step forward by investigating (a) the agreement between SUN and BUN and (b) the diagnostic performance of SUN to discriminate more advanced AKI from less severe cases longitudinally over a time period of up to 8 days.

Methods

Study Design

This international, multicenter, prospective cohort study enrolled patients diagnosed with AKI as per the AKI Network (AKIN) criteria [7] upon first presentation in the respective medical facility. Data were obtained by several investigators at Beth Israel Medical Center, New York, USA; Hans Dieter Schmidt Regional Hospital, Joinville, Santa Catarina, Brazil; and University Cajuru Hospital, Curitiba, Brazil. Institutional Review Boards of all involved institutions have approved the study and the merging of the databases in expedited review.

Measurements

Unstimulated saliva was collected within 4 h of blood collection. Subjects were asked to refrain from drinking and eating for at least 15 min prior to saliva collection. Saliva was collected in a plastic cup and approximately 50 µl of saliva was used to moisten the colorimetric SUN dipstick (Integrated Biomedical Technology, Elkhart, Ind., USA). After 1 min, the color of the test pad was compared to 6 standardized color fields indicating SUN concentrations of 5–14 (color pad #1), 15–24 (#2), 25–34 (#3), 35–54 (#4), 55–74 (#5), and ≥75 (#6) mg/dl, respectively [5].

Statistical Analysis

Parametric data are presented as mean ± standard deviation (SD) and non-parametric data are presented as median and interquartile range (IQR). The agreement between SUN and BUN was tested employing the analysis of variance with a post hoc Bonferroni correction for multiple testing. Differences between BUN and SUN (transformed to a continuous variable by choosing the midpoint for each range) were displayed as error bars (with 95% CIs) at different days and depicted as a modified Bland–Altman plot using BUN as the 'gold-standard' [8, 9]. Agreement between SUN and BUN over the entire period was also tested by the development of linear mixed effects models, which assumes different random intercepts for each subject and random slopes for each day-to-day period to account for variations between the days where measurements were conducted. Diagnostic performance of SUN and BUN to discriminate AKIN III vs. earlier stages was analyzed by predictive values (i.e. sensitivity and specificity after defining AKIN III as the binary outcome), and the area under the receiver operating characteristics curve at each of the 4 observation days following the approach defined by Calice-Silva et al. [6].

A two-sided p value <0.05 was considered statistically significant. Analyses were done in R 3.2.1 (codename 'World-Famous Astronaut'; R Foundation for Statistical Computing, Vienna, Austria) additionally using the packages plyr, sandwich, nlme, multcomp, pROC and ggplot2 [10].

Results

Patient Population

Forty patients were recruited; 3 of them had insufficient documentation and therefore 37 were studied in the primary analysis analyzing the agreement between SUN and BUN. Patients were followed over a variable length of time (median 4 (IQR 2–6.8) days), limited by a maximal observation period of 8 days. For the secondary analysis assessing the diagnostic performance of the SUN dipstick, we had to restrict the analysis to 31 subjects because

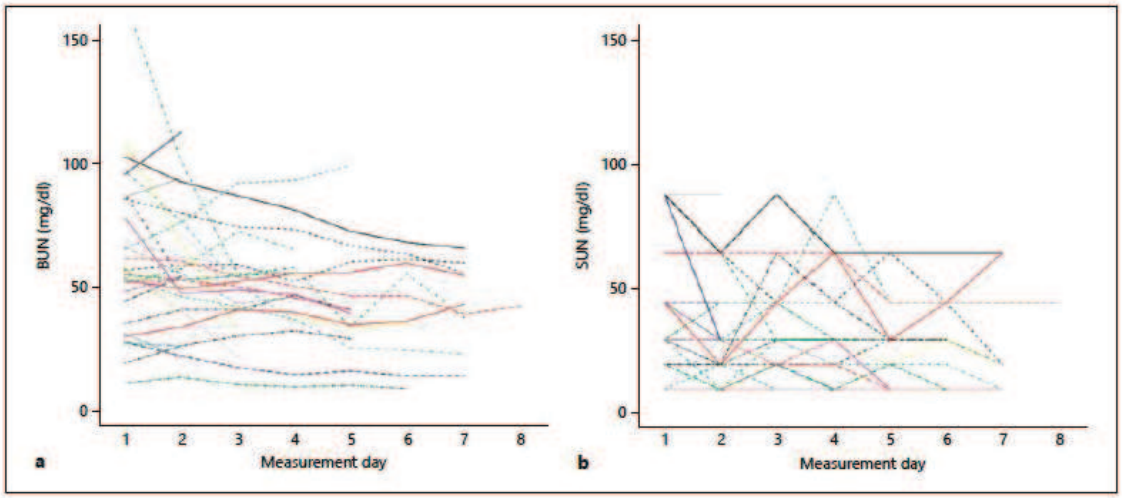

Fig. 1. Line plots depicting BUN (**a**) and SUN (**b**) at all observation days.

Table 1. Demographic and treatment-related characteristics of all studied subjects

	Number, count	Age, years	Weight, kg	Male gender, count (%)	Black race, count (%)	SUN, mg/dl	BUN, mg/dl	Creatinine, mg/dl	Pre-renal etiology	Hemodialysis
All subjects	37	60±19.1	75.7±21.1	22 (59.5)	31 (51.6)	37±26.8	61.9±30.9	4.1±3.3	n/a	n/a
AKIN 1*	11	66.3±18.5	82.3±21.9	6 (54.5)	9 (13.6)	24±12.7	46.2±16.9	2.2±1	6 (54.5)	0 (0)
AKIN 2*	6	67.3±13.8	60.3±12.4	5 (83.3)	5 (7.4)	22±13.3	51.8±20.6	2.4±0.4	4 (66.7)	0 (0)
AKIN 3*	14	53.4±21.9	76.4±17.6	11 (78.6)	13 (24.3)	55.9±27.9	80.2±34.8	6.2±4	4 (28.6)	2 (14.3)

* Data on AKIN stages (and various other parameters) missing from BIMC subjects (n = 6). n/a = Not available.

of missing data. The characteristics of all patients are shown in table 1. It is of note that SUN, BUN and creatinine were substantially higher for those classified as AKIN III compared to the less severe AKIN stages I and II (table 1).

SUN and BUN

From an elevated baseline level for most patients, there was a clear and discernible trend toward a paralleling decrease in both parameters (fig. 1). A consistent decrease in serum creatinine can be seen in online supplemental figure 1 (for all online suppl. material, see www.karger.com/doi/10.1159/000445041). Table 2 shows a consistent underestimation of BUN by the SUN (ranging from 17.2 (day 4) to 25.7 (day 2)), which is also seen in figures 2 and 3. Pooling all data, regardless of the observation day, and analyzing BUN stratified by measured SUN categories showed

Table 2. Test results of BUN and SUN on observation days 1 through 4

	Number, count	SUN, mg/dl	BUN, mg/dl
All subjects	120	32.6±22.5	55±24.9
Day 1	37	37±26.8	61.9±30.9
Day 2	36	30.3±20.2	56±22.6
Day 3	24	31.1±19.7	49.5±19.7
Day 4	23	30.9±21.6	48.1±20.2

significant differences between testpads 1 and 2 and testpads 3, 4, 5 and 6; and significant differences between testpads 3, 4 and 5 and testpad 6 (table 3). Table 3 shows a consistent underestimation of BUN by SUN, which is also seen in figure 2. Plotting the differences as a function of BUN and

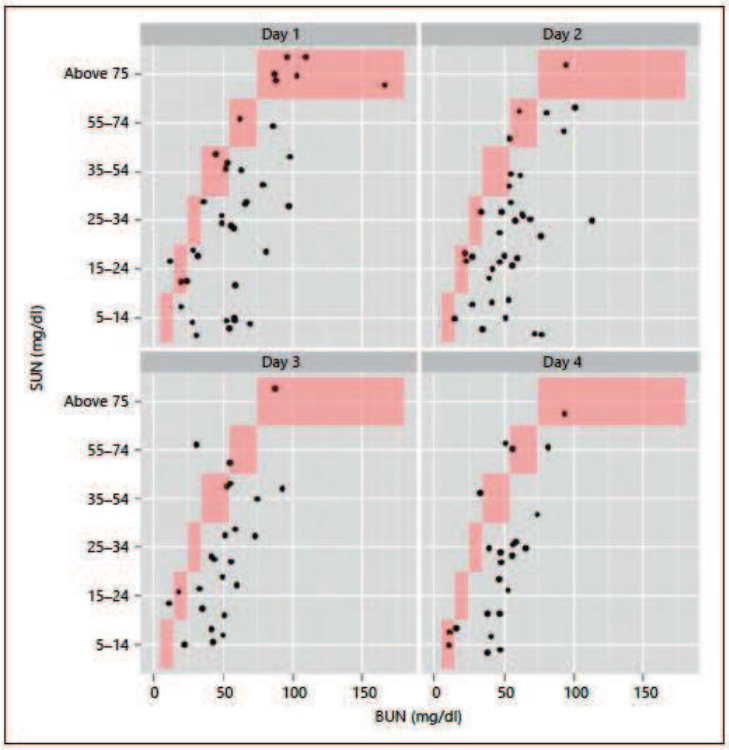

Fig. 2. Scatter plot showing the relationship between SUN and BUN.

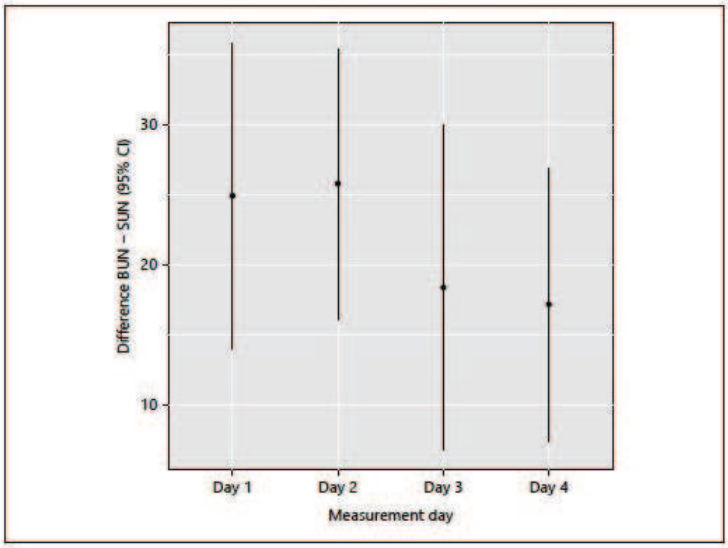

Fig. 3. Average systematic bias between BUN and SUN (quantified as the midpoint of the categories of the dipstick) at the first 4 observation days.

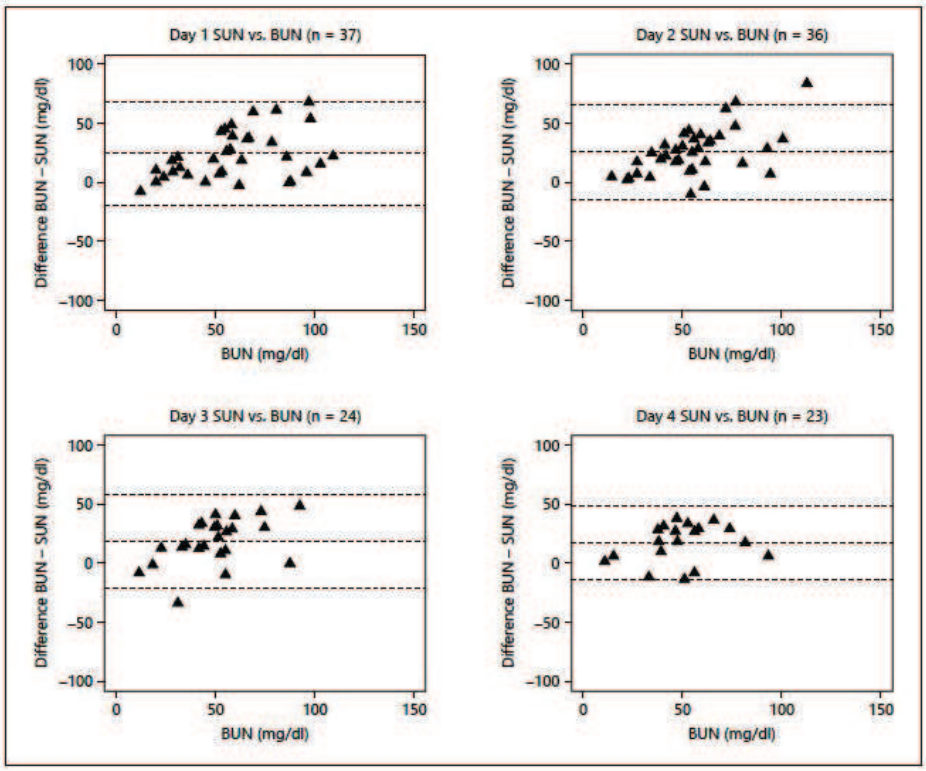

Fig. 4. Bland–Altman plot depicting the systematic bias between SUN and BUN as a function of the BUN as the reference method.

Table 3. Test results of BUN (all patients at all observation days) stratified as per SUN

SUN testpad*	Testpad color	Tests, n	BUN, mg/dl**
1 (5–14; 9.5)		26	41.4 (27.3–53.2)[3–6]
2 (15–24; 19.5)		27	39.2 (25.4–50.5)[3–6]
3 (25–34; 29.5)		31	56 (47.9–64.9)[1, 2, 6]
4 (35–54; 44.5)		15	55.1 (53–74.3)[1, 2, 6]
5 (55–74; 64.5)		12	61.5 (54.8–82.8)[1, 2, 6]
6 (>75; 87.5)		9	94.4 (87.9–103)[1–5]

* Values are SUN range; midpoint (mg/dl).
** Data presented as median (25^{th} and 75^{th} percentile).
[1] $p < 0.05$ (vs. testpad 1); [2] $p < 0.05$ (vs. testpad 2); [3] $p < 0.05$ (vs. testpad 3); [4] $p < 0.05$ (vs. testpad 4); [5] $p < 0.05$ (vs. testpad 5); [6] $p < 0.05$ (vs. testpad 6).

as a modified Bland–Altman plot showed consistent non-significant biases and proportional errors (fig. 4).

BUN stratified by measured SUN categories furthermore showed significant differences between testpad 1 and testpads 3, 4, 5 and 6 and significant differences between testpads 3, 4 and 5 and testpad 6. While these differences imply the ability to diagnose elevated SUN (and consequently BUN) with sufficient diagnostic accuracy, the lack of difference at lower levels also suggests a lower accuracy when SUN and BUN are low. One possible interpretation is that the dipstick appears to be a useful tool to diagnose very high SUN but not the lower levels (as often found in newly developing AKI), where it may misdiagnose and result in false-negative results. Currently, this is a limitation that may be addressed in future developments of the method.

Development of two linear mixed effects models, one with random intercept and another with random inter-

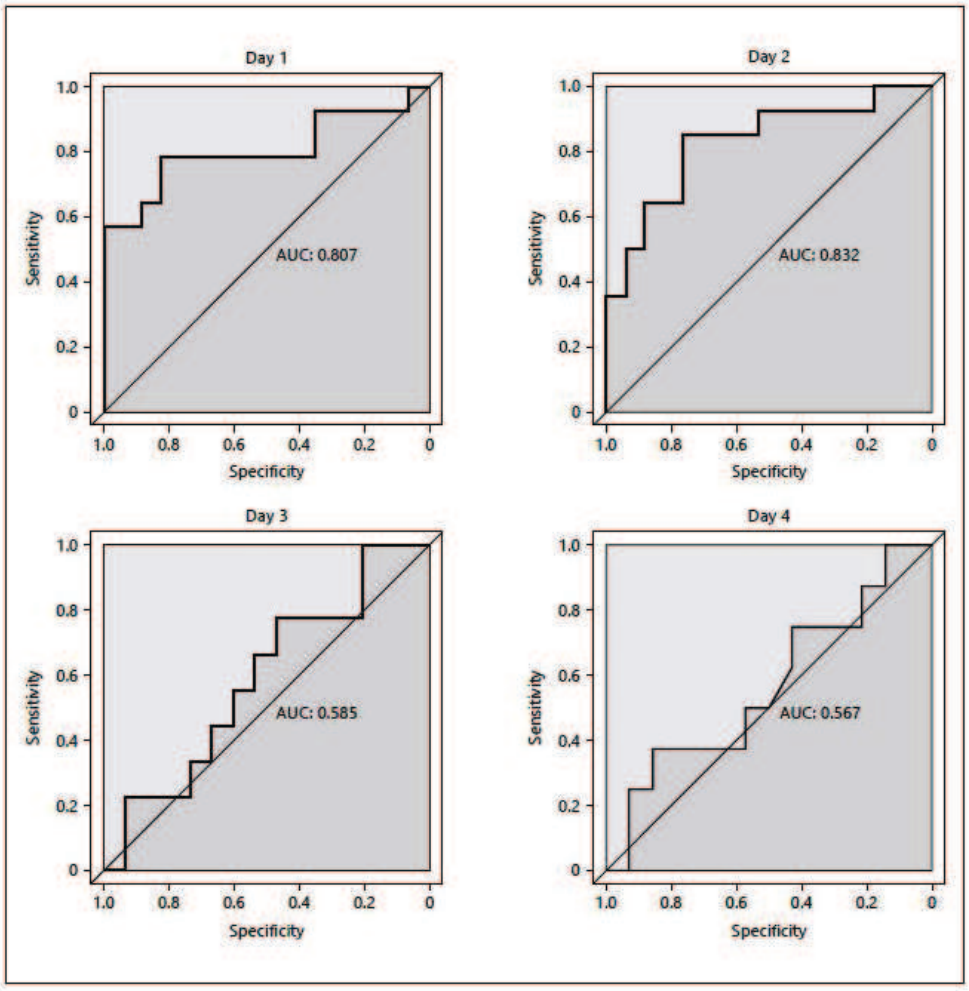

Fig. 5. Receiver operating characteristics curve of BUN to discriminate AKI at stage 3 as per the AKIN criteria.

cept (individual patient) and slope (individual observation day), with SUN as the dependent variable and BUN as fixed effects, was not significantly different in the likelihood ratio test. BUN was a significant fixed effect of SUN in both mixed effects models (0.52 mg/dl SUN per mg/dl BUN; $p < 0.001$ with an intercept of 3.7 and 3.2 mg/dl, respectively; $p = 0.42$).

Evaluation of the Diagnostic Performance

Evaluating the diagnostic performance of BUN and SUN, respectively, showed both parameters, applying the criteria of Calice-Silva et al. [6], enabling the discrimination of AKIN III vs. AKIN I and II on all observation days. The area under the receiver operator characteristics curve decreased from 0.81 (95% CI 0.63–0.98) on observation day 1 to 0.57 (95% CI 0.63–0.98) on observation day 4 (fig. 5) for BUN. For SUN, the area under the receiver operator characteristics curve decreased from 0.85 (95% CI 0.71–0.98) on observation day 1 to 0.63 (95% CI 0.63–0.98) on observation day 4 (fig. 6). It is of note that the predictive value of serum creatinine did not change during the observation period (0.90 (95% CI 0.76–1.00) on observation day 1 to 0.91 (95% CI 0.76–1.00) on observation day 4 (online suppl. fig. 2)).

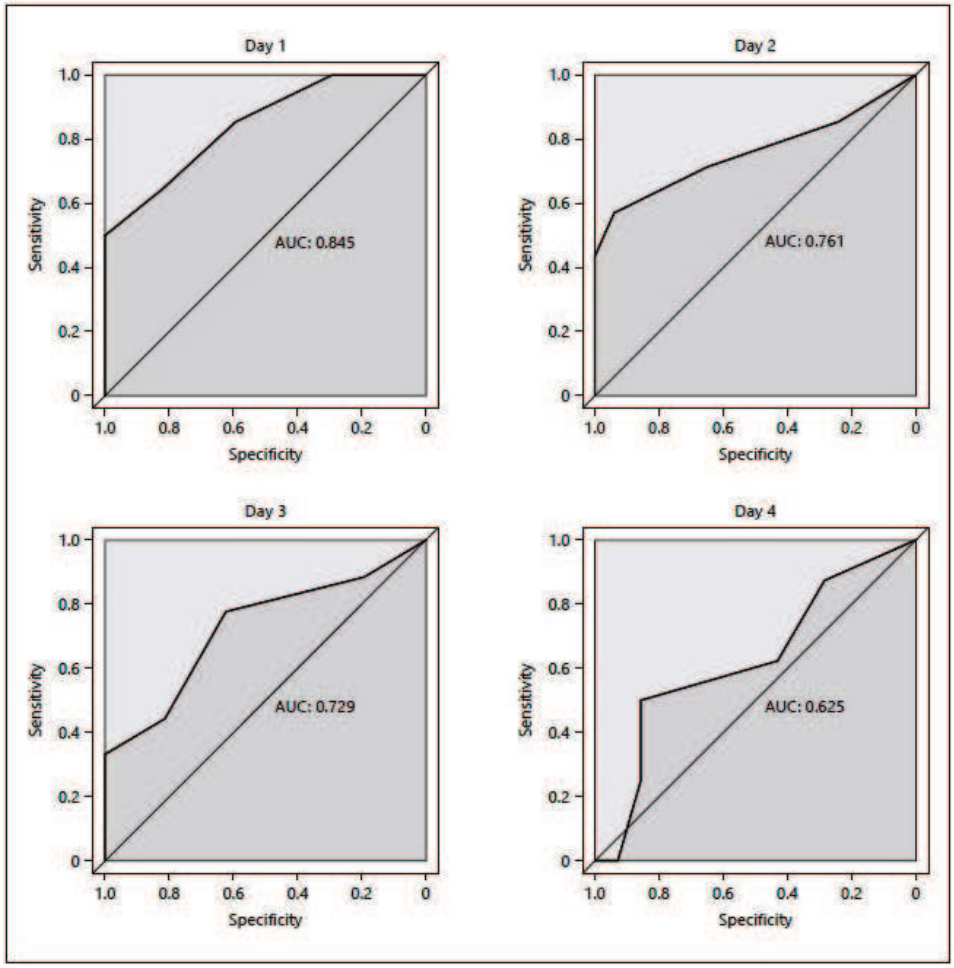

Fig. 6. Receiver operating characteristics curve of SUN to discriminate AKI at stage 3 as per the AKIN criteria.

Discussion

These analyses confirm our earlier reports on the agreement between SUN and BUN [5, 6] and carry current knowledge on the diagnostic performance of SUN an additional step forward by analyzing the agreement and the diagnostic utility of SUN over a prolonged period of time (4 days) following first AKI diagnosis (as per the AKIN criteria [7]). We have found that the agreement between BUN and SUN was comparable on all observation days, generally following a declining trend in BUN and creatinine (fig. 1 and online suppl. fig. 1). While there is a consistent underestimation of BUN, SUN does, particularly at higher levels (>50 mg/dl), reliably reflect BUN and follows its changes over time (fig. 2–4). Table 3 shows a consistent underestimation of BUN by SUN, which decreases as BUN rises, which is also seen in figure 2. This is also seen in the systemic bias as shown in figure 4; however, for the purpose of discrimination of levels indicating the presence of AKI at AKIN III, where BUN is higher, the underestimation appears not to affect diagnostic performance when using SUN.

SUN in our data was consistent with the report of Calice-Silva et al. [6]; it was able to discriminate AKI at AKIN

stage III vs. the less severe stages I and II, with a diagnostic performance comparable to BUN on every analyzed observation day (fig. 5 and 6). As to be expected, the diagnostic performance of both parameters decreased during the course of the study, likely because of the response to treatment; however, the performance of SUN was consistent with BUN on every observation day and discriminated with comparable accuracy (fig. 5 and 6).

Our data suggest that irrespective of the timing of diagnosis with regard to the onset of AKI, high SUN should immediately cause concern and trigger AKI-specific measures such as removal of nephrotoxins, assessment of fluid status, treatment of the underlying disease (e.g. infection), and possibly transfer to the next higher level of healthcare for more specific supportive measures, such as temporary dialysis. Furthermore, given the consistent agreement on all observation days, the dipstick appears to be a suitable tool to follow treatment progression. Differences of salivary flow rate in terms of age, gender, race and socioeconomic factors are a drawback of the dipstick method [11, 12]. Given the considerably small sample size, interpretation must be made with caution; also, the fact that measurements were obtained from Brazil and the United States, and that 52% of the study participants were of black race lead to a result with some degree of external validity. Inter-observer variability was not investigated in the current analysis; however, based on our earlier results, these effects can be considered negligible [5]. In addition to well-known limitations of BUN as a marker of AKI, limitations of the SUN dipsticks such as the underestimation of BUN at levels below 50 mg/dl, with, the proportional error decreasing with the increase in BUN and the occurrence of false negative assessments are recognized. However, there are major strengths to this technique that need to be outlined. Currently, available POCT devices are expensive and, in addition, the test materials required for each measurement are expensive. In addition enzyme-containing cartridges need controlled cooled storage. Furthermore, the operating temperature maxima of many devices are exceeded by the ambient temperature in many developing countries. While in centralized healthcare facilities these devices are valuable, in remote areas with limited resources, lack of electricity, absence of a clean and ventilated/air-conditioned environment and lack of adequate training of involved personnel are obstacles to routine use. Considering the low cost of less than 1 US dollar (compared to around 7$ for a BUN measurement in the US) of the SUN dipstick, the remarkable ease of use and the low level of inter-observer variability, it is a particularly useful and promising tool for the diagnosis and follow-up of AKI especially in places where facilities are absent.

Conclusions

In view of the currently launched global efforts to eradicate mortality due to reversible AKI by the '0by25 Initiative' of the ISN, there is great interest in developing new diagnostic tools to diagnose AKI and disease severity. We believe that the SUN dipstick could be of a great utility in the diagnosis of AKI and the evaluation of disease severity, particularly in the most remote settings.

Acknowledgments

We acknowledge the generous provision of SUN dipstick free of charge by Integrated Biomedical Technology. The results presented in this paper have not been published previously in whole or part, except in the abstract form at the LI ERA-EDTA 2014 congress in Amsterdam, the Netherlands.

Funding

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Disclosure Statement

Dr. V. Calice-Silva was a fellow of the ISN and received scholarship from the Brazilian Government (CAPES) during part of the time when the study was conducted. Drs. J. Callegari, M. Carter, P. Kotanko and N.W. Levin hold stock in Fresenius Medical Care. All other authors have no financial interests to declare.

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4.3. Study 3: Diagnostic performance of a Saliva Urea Nitrogen Dipstick to detect Kidney Disease in Sub-Sahara Africa – in manuscript form.

TITLE PAGE

Title

Diagnostic performance of a Saliva Urea Nitrogen Dipstick to detect Kidney Disease in Sub-Sahara Africa

Authors

[a,b,c]Rhys Evans*, [d,e]Viviane Calice-Silva*, [f]Jochen G. Raimann, [a,b]Ulla Hemmila, [b]Alison Craik, [b]Mwayi Mtekateka, [b]Fergus Hamilton, [b]Zuze Kawale, [c]Hamish Dobbie, [g]Gavin Dreyer, [h]Nathan Levin, [f]Peter Kotanko, [d]Roberto Pecoits-Filho.

In collaboration with the International Society of Nephrology (ISN) 0by25 Initiative

Affiliations

[a]College of Medicine, Malawi, [b]Nephrology Dept. Queen Elizabeth Central Hospital, Malawi, [c]Dept. of Renal Medicine and Transplantation, Bart's Health, London, UK; [d]School of Medicine, Pontifícia Universidade Católica do Paraná, Curitiba and [e]Pró-rim Foundation, Joinville, Brazil; [f]Research Division, Renal Research Institute, New York , USA; [g]Nephrology Dept. Royal Free Hospital, London, UK; and, [h]Icahn School of Medicine at Mount Sinai, New York, USA.

Key words: acute kidney injury, kidney disease, salivary urea nitrogen, dipstick, diagnostic tool, and low-resource setting.

* Both authors contributed equally.

Corresponding author:

Rhys D R Evans, MRCP

Department of Renal Medicine and Transplantation, Bart's Health, Royal London Hospital, London, UK

Rhys.Evans@bartshealth.nhs.uk; rhysdrevans@gmail.com

ABSTRACT

Background: Kidney disease (KD), including Acute Kidney Injury (AKI), is common, severe and leads to significant mortality in the developing world. However, simple tools to facilitate diagnosis and guide treatment are lacking. We studied the diagnostic performance of saliva urea nitrogen (SUN) measured by dipstick to diagnose KD in a low-resource setting.

Methods: Medical admissions to a tertiary hospital in Malawi had serum creatinine (sCR) tested at presentation; SUN was measured using a dipstick. Patients with sCR above normal range underwent serial measurements of SUN and blood urea nitrogen (BUN) for up to 7 days. Hospital outcome was recorded in all.

Findings: 742 patients were included (age 41±17·3 years, 56·1% male); 146 (19.7%) had KD, including 114 (15.4%) with AKI. SUN >14mg/dL had a sensitivity of 0·72 and a specificity of 0·87 to diagnose KD; specificity increased to 0·97 when SUN levels were combined with self-reported urine output. The diagnostic performance of SUN was comparable to the one of BUN (SUN Area Under Curve [AUC] 0·82, 95% Confidence Interval [CI] 0·78–0·87; BUN AUC 0·82, 95% CI 0·59–1·0). SUN >14mg/dL on admission was an independent predictor of all-cause mortality (HR=2·43 [95% CI 1·63–3·62]).

Interpretation: SUN measured by dipstick can be used to identify patients with KD in a low-resource setting. SUN is an independent predictor of mortality in this population.

Funding: International Society of Nephrology; Royal Society of Tropical Medicine and Hygiene.

INTRODUCTION

Acute kidney injury (AKI) is common worldwide, causing high morbidity and mortality, particularly in the developing world (1, 2). Here, AKI primarily affects young patients with limited comorbidity, is predominantly community-acquired, and is commonly caused by infective illnesses, volume depletion, and nephrotoxicity (3, 4).

Many deaths resulting from AKI in low resource settings (LRS) may be preventable (5). However, a number of major challenges exist when managing AKI in these areas: a) a disparity in health care resources available in urban compared to rural areas; b) poor awareness among patients and healthcare workers of AKI and the need for its early detection and treatment; c) a scarcity of trained personnel and resources for renal replacement therapy; and d) a lack of reliable and cost-effective tools to diagnose AKI (5, 6).

The lack of medical and laboratory infrastructure in LRS make the development of an inexpensive, non-invasive, and reliable bedside diagnostic tool to identify patients with kidney disease, including AKI, essential (5). The salivary urea nitrogen (SUN) dipstick has been suggested as a potential screening tool for acute and chronic kidney disease (7, 8). Indeed, in our previous studies in developed settings, SUN strips demonstrated good diagnostic performance to detect kidney dysfunction, especially at the higher levels of blood urea nitrogen (BUN) (7). In the present study we aimed to explore the diagnostic performance of this tool in a LRS, where access to laboratory measurements of renal function are often limited, and where the SUN test would be of greatest value.

METHODS

Study design, setting and participants

We conducted a prospective observational study at Queen Elizabeth Central Hospital (QECH) in Blantyre, Malawi, one of the poorest countries in the world. QECH acts as both a district hospital and a tertiary hospital for the Southern region of Malawi, although the majority of patients admitted are from Blantyre district itself, population approximately 1 million.

All patients aged 14 years or older admitted to the general medical wards between 27th April and 17th July 2015 were eligible. Patients unable to give informed consent, patients transferred to the medical ward from another ward or hospital, and patients who were unable to collect sufficient volume of saliva (including patients with significantly reduced level of consciousness) were excluded from the study.

Patients enrolled were screened for kidney disease with serum creatinine (sCR) measurement. Concomitantly, we measured salivary urea nitrogen (SUN) levels using a dipstick (Integrated Biomedical Technology, Elkhart, Ind., USA). Patients with sCR above the local laboratory reference range (>90μmol/l in women; >104μmol/l in men) were managed by the nephrology team and followed for a period of up to 7 days or until hospital discharge if sooner. During this period we collected serial saliva and blood samples for measurement of SUN, BUN and sCR. Demographic and clinical data, including signs and self-reported symptoms (increased thirst and reduced urine output) of altered volume status, were also recorded.

Data Collection

Saliva and blood samples were collected simultaneously on admission (day 0) for the measurement of SUN and sCR, and then at 24 hours (day 1) and every 48 hours thereafter (days 3,5, and 7), for the measurement of SUN, BUN and sCR.

SUN measurement

Subjects were asked to refrain from drinking and eating for at least 15 min prior to saliva collection. Unstimulated saliva was collected in a plastic cup and approximately 50µl of saliva were used to moisten the test pad of a colorimetric SUN dipstick. After 1 min, the colour of the test pad was compared to 6 reference pads indicating increasing SUN concentrations: 5–14mg/dL (pad #1), 15–24mg/dL (pad #2), 25–34mg/dL (pad #3), 35–54mg/dL (pad #4), 55–74mg/dL (pad #5), and ≥ 75mg/dL (pad #6) mg/dl (figure S1– Supplementary material) (8).

Serum creatinine (sCR) and urea measurement

sCr was measured by Jaffe method (9) and BUN by the urease method (10) [either by Flexor Junior Clinical Chemistry Analyzer (Vital Scientific, Dieren, The Netherlands) or by Mindray Chemistry Analyzer BS-120 (Shenzen Mindray Bio-Medical Electronics Company, Shenzen, China)] in a local laboratory.

Definitions

AKI, Acute Kidney Disease/Disorder (AKD) without AKI, and Chronic Kidney Disease (CKD) were diagnosed and staged by KDIGO (Kidney Disease Improving Global Outcomes) criteria (1, 11). Kidney Disease (KD) is used to refer to AKI, AKD without AKI, and stable CKD. AKD incorporates both confirmed AKI and AKD without AKI (figure S2– Supplementary material).

The study was approved by the institution's ethics committee (P.11/14/1660) and was conducted in accordance with the Declaration of Helsinki and good clinical practice.

Outcome measures

The primary outcome measure was the diagnostic performance of the SUN dipstick to detect KD, alone and in combination with self-reported changes in urine output and thirst. Secondary outcome measures were the agreement of

SUN with BUN at presentation and during management of KD, and the ability of SUN to predict in-hospital mortality in this population.

Statistical analyses

For the statistical analyses, urea results were converted to BUN (mg/dL) and SUN was transformed to a continuous variable by choosing the midpoint for each range. Descriptive statistics were presented as mean ± standard deviation (SD) and as median and interquartile range (IQR), depending on data distribution.

The agreement between SUN and BUN was tested by one-way analysis of variance (ANOVA) with a post hoc Bonferroni correction for multiple testing. Differences between BUN and SUN (midpoint concentration of the respective test pad range) were displayed as error bars (with 95% confidence intervals [CI]) at different days and represented as a modified Bland–Altman plot using BUN as the reference method (12). Agreement between SUN and BUN over the entire period was also tested using linear mixed effects models with random intercepts for each subject and random slopes for each two-day period.

Diagnostic performance of SUN and BUN to detect KD was analyzed using sensitivity and specificity, and by the area under the receiver operating characteristics (ROC) curve at each of the observation days. Optimal diagnostic thresholds were determined based on the maximum Youden's index (Youden's index= sensitivity + specificity– 1). In addition, sensitivity and specificity were used to evaluate the diagnostic performance of SUN to detect KD combined with the two self-reported clinical parameters, namely: a) increased thirst and, b) reduced urine output (UO)(13).

Cox proportional hazard models were constructed to evaluate the predictors of death in this population. Age, sex, the presence of diabetes, and SUN levels were assessed. Kaplan-Meier curves were used to assess the mortality risk in patients stratified by the absence or presence of SUN levels >14 mg/dL (i.e. SUN

> test pad #1). All surviving patients were censored at Day 15.

A two-sided p value <0·05 was considered statistically significant. Analyses were done using R 3.2.1 (codename ‘World-Famous Astronaut’; R Foundation for Statistical Computing, Vienna, Austria) (14).

RESULTS

Cohort description

Out of 892 medical patients admitted to the hospital during the study period, 742 had both sCR and SUN measurements and were thus included in the analysis (Figure 1). The mean age was 41±17 years old, 56% were male. 146 (19.7%) patients had kidney disease: 114 (15.4%) AKI, 26 (3.5%) AKD without AKI, and 6 (0.8%) stable CKD. AKI was stage 3 in 67 (58.8%) patients (Table 1).

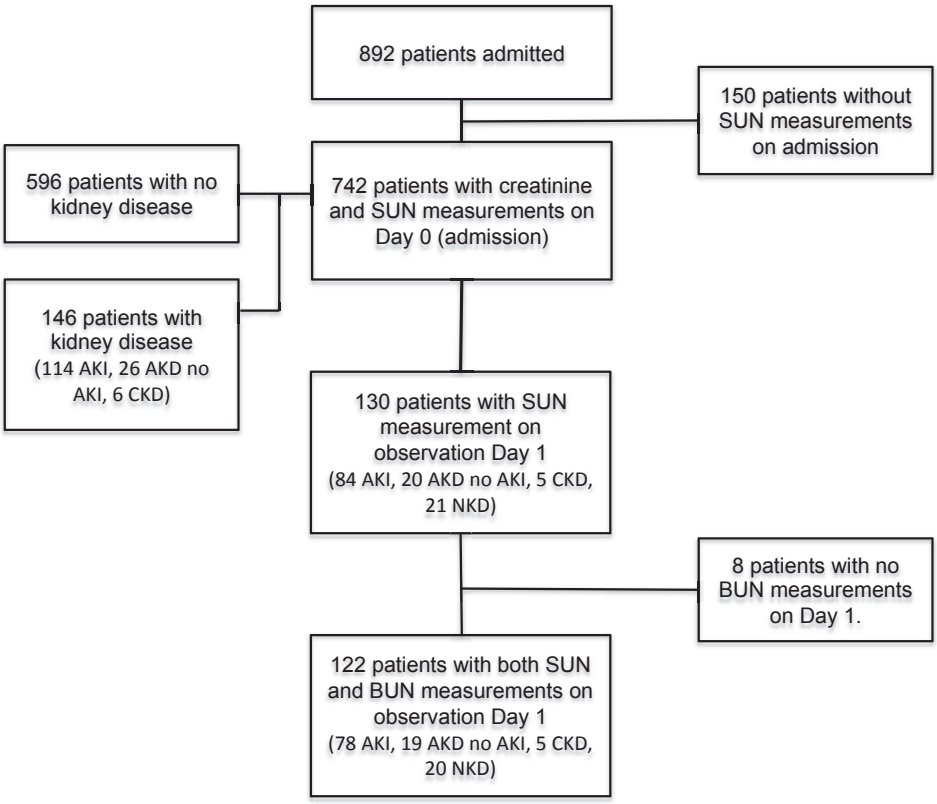

Figure 1: Cohort Description
SUN – Saliva Urea Nitrogen; AKI – Acute Kidney Injury; AKD – Acute Kidney Disease/Disorder; CKD – Chronic Kidney Disease; NKD – No Kidney Disease; BUN – Blood Urea Nitrogen

Table 1: Demographics, SUN and serum creatinine levels on admission

	N [count]	Age [years]	Male gender [%]	SUN [mg/dL]	Creatinine [micromol/L]
No kidney disease	596	40·1±17·2	55·37	11·1±4·8	62·6±20·5
AKI stage 1	26	45·9±16·2	38·46	28·2±24·5	140·7±1·7
AKI stage 2	21	36·5±13·9	61·9	34±26·6	239±111·9
AKI stage 3	67	44·4±18	67·16	53±32·6	811·6±704·2
AKD with no AKI	26	47±17	53·85	25·3±27·6	342·3±525·9
CKD	6	47·7±23·4	66·67	37·5±28·4	886·5±867·3
All	742	40·8±17·3	56·06	16·8±18·6	156±335·8

AKI: Acute kidney injury – KDIGO stages 1, 2 and 3; AKD without AKI: Acute kidney disease/disorder without AKI; CKD: Chronic Kidney disease; SUN: Saliva Urea Nitrogen; BUN: Blood Urea Nitrogen.

130 patients had SUN measured on day 1. The characteristics of these patients are shown in Table 2. There were significantly more patients with hypovolemia as determined by the study team, self-reported reduced urine output and increased thirst (60·5%, 29·6% and 100% respectively) in the group with AKI stage 3 compared to the other groups (Table S1– Supplementary material).

Table 2: Demographics and biochemical findings (SUN, BUN and serum creatinine) in patients who had SUN measured on day 1

	N [count]	Age [years]	Male gender [%]	SUN [mg/dL]	BUN [mg/dL]	Creatinine [micromol/L]
No kidney disease	21	42±20·3	71·43	15·7±5·9	32·4±35·6	100·1±23·6
AKI stage 1	23	45·5±16·7	39·13	23·7±22·6	37·7±21·4	130·5±44·4
AKI stage 2	17	36·5±10·8	58·82	22·7±8·5	35·9±19·1	193·9±-91·7
AKI stage 3	44	41·1±16·3	72·73	46·4±-31·7	92·4±44·8	760·1±761·6
AKD with no AKI	20	45·1±16·2	55	24·8±24	45·3±34·5	309±442·7
CKD	5	50·2±25·3	60	25·5±5·5	75·7±58·1	820·2±970·6
All	130	42·3±6·8	61·54	30·2±25·8	57·7±44·2	403·3±583·4

AKI: Acute kidney injury – KDIGO stages 1, 2 and 3; AKD without AKI: Acute kidney disease/disorder without AKI; CKD: Chronic Kidney disease; SUN: Saliva Urea Nitrogen; BUN: Blood Urea Nitrogen.

SUN and BUN measurements

When comparing results of both parameters, there was a similar trend over time (Figure S3– Supplementary material), with SUN consistently underestimating BUN in the follow-up period (Figure 2). However the agreement between BUN and SUN appears unaffected by treatment and progress of care (Figure 3). Plotting the differences (BUN – SUN midpoint) as a function of BUN and as a modified Bland–Altman plot showed consistent non-significant biases and proportional errors (Figure 4).

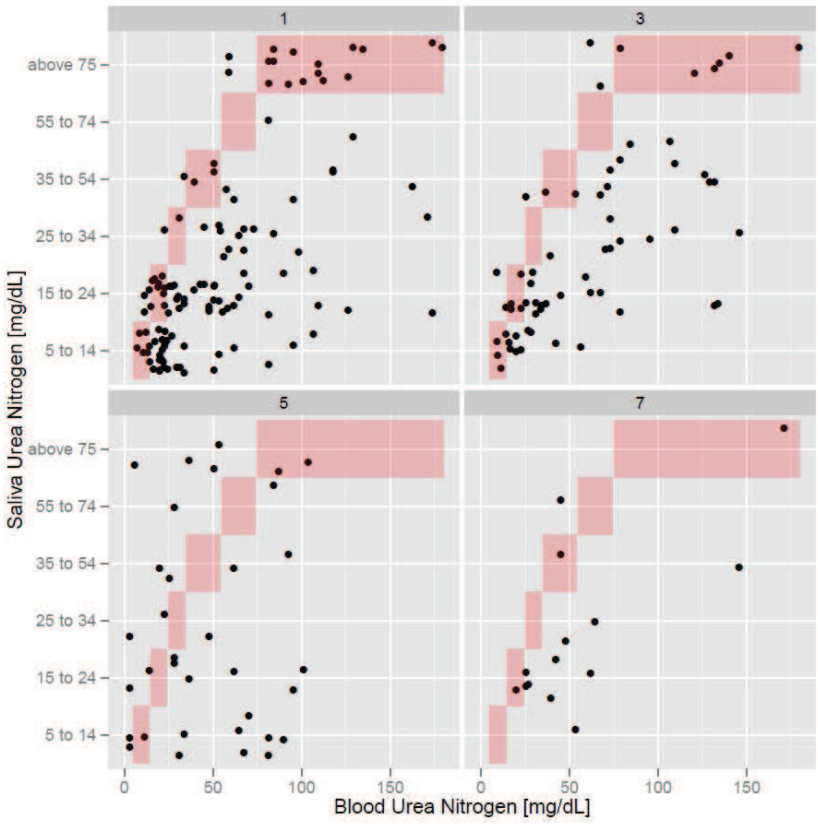

Figure 2: Scatterplot demonstrating the relationship between SUN and BUN from day 1 to day 7.

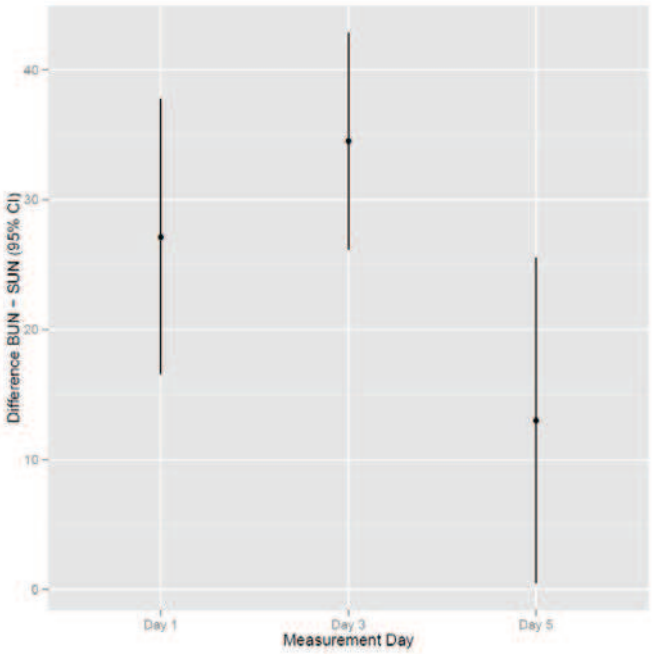

Figure 3: Point estimates and 95% confidence intervals of the difference between BUN and SUN (quantified as the midpoint of the categories of the dipstick) on days 1,3 and 5.

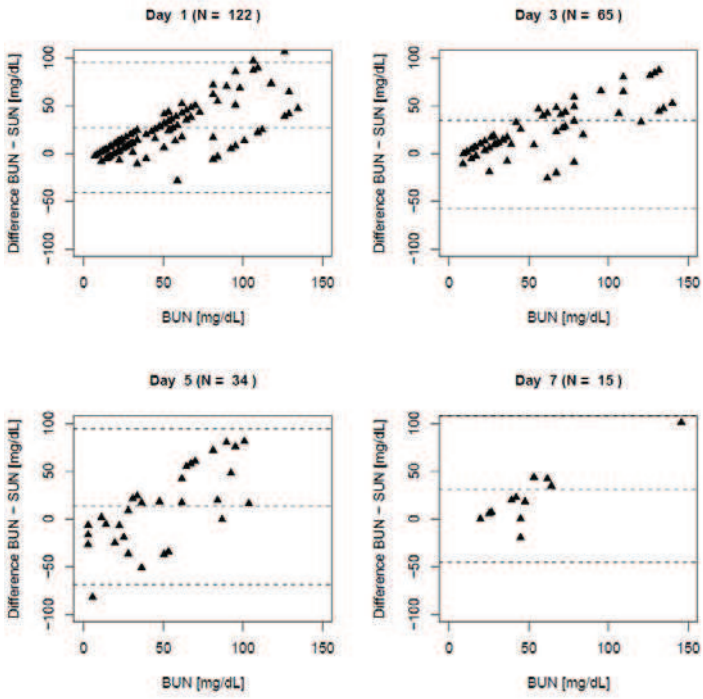

Figure 4: Bland–Altman plot indicating the difference between BUN and SUN as a function of the BUN on days 1, 3, 5 and 7. Dotted lines represent the mean difference +/- 2 standard deviations at each observation day. The data indicate a consistent systematic and proportional bias consistent throughout the observation period.

BUN stratified by SUN categories showed significant differences between test pad 1 and test pads 3, 4, 5 and 6, between test pads 2, 3 and 4 and test pad 6 and between test pad 2 and test pad 4 (Table S2 – Supplementary material). These differences imply the ability to diagnose elevated BUN with sufficient diagnostic accuracy; the absence of significant difference at lower levels of SUN (e.g. between test pad 1 and 2, or between 2 and 3) indicates lower accuracy of SUN at lower levels of BUN.

The two linear mixed effects models, one with random intercept and another with random intercept (individual patient) and slope (individual observation day), with SUN as the dependent variable and BUN as fixed effects, were not significantly different in the likelihood ratio test. BUN was a significant fixed effect of SUN in both mixed effects models (0·26 mg/dl SUN per mg/dl BUN; $p < 0·001$).

Diagnostic performance of SUN and BUN

The area under the receiver operator characteristics curve for SUN to detect KD decreased from 0·82 (95% CI 0·78–0·85) on observation day 0 (screening day) to 0·69 (95% CI 0·50–0·78) on observation day 1 (Figure 5). Sensitivity and specificity were highest at day 0 (0·71 and 0·87 respectively). The optimal diagnostic threshold for SUN to diagnose kidney disease was test pad #2. For BUN, the area under the receiver operator characteristics curve was 0·82 (95% CI 0·59–1·0) on day 1 (Figure 6). Self-reported increased thirst analyzed combined with SUN > 14mg/dL (SUN > test pad #1) did not show significant improvement on sensitivity and specificity to detect KD. When analyzing self-reported decreased urine output combined with SUN > 14mg/dL (SUN > test pad #1) to diagnose KD significant differences in the sensitivity (0·22) and specificity (0·97) were demonstrated. While sensitivity is low, the specificity increased by 0·1 up to 0·97 when self-reported information on urine output was added.

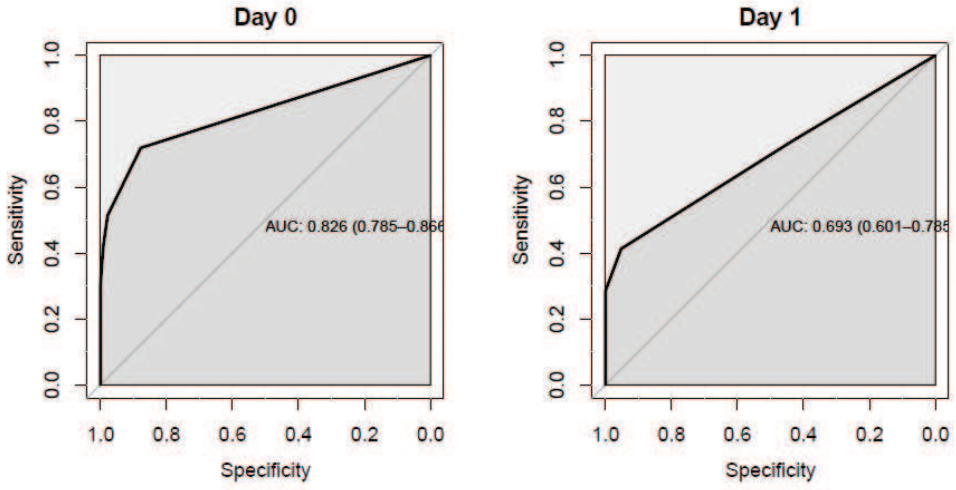

Figure 5: Receiver operating characteristic (ROC) curves of SUN to Kidney Disease (AKI, AKD without AKI, and CKD) on days 0 and 1.

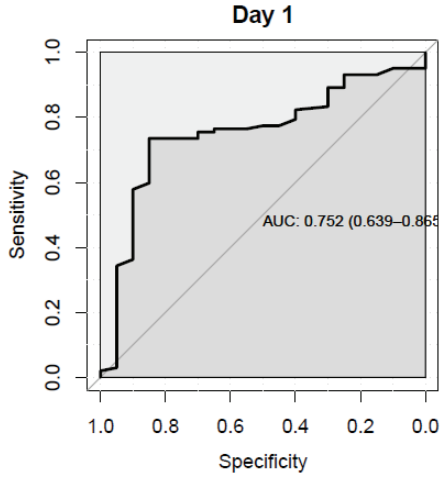

Figure 6: Receiver operating characteristic (ROC) curves of BUN to detect Kidney Disease (AKI, AKD without AKI, and CKD) on day 1.

Outcome analyses

Hospital outcome was determined in 702 patients. 104 (14·8%) of these patients died, with 17 (2.4%) doing so after the censoring at day 15. Age, gender, the presence of diabetes and SUN results were evaluated as predictors of time to in-hospital death; elevated SUN >14mg/dL (SUN > test-pad > #1) was the only factor which was an independent predictor of all cause mortality, HR=2·23 [95%

CI 1·50 to 3·32] (Figure 7).

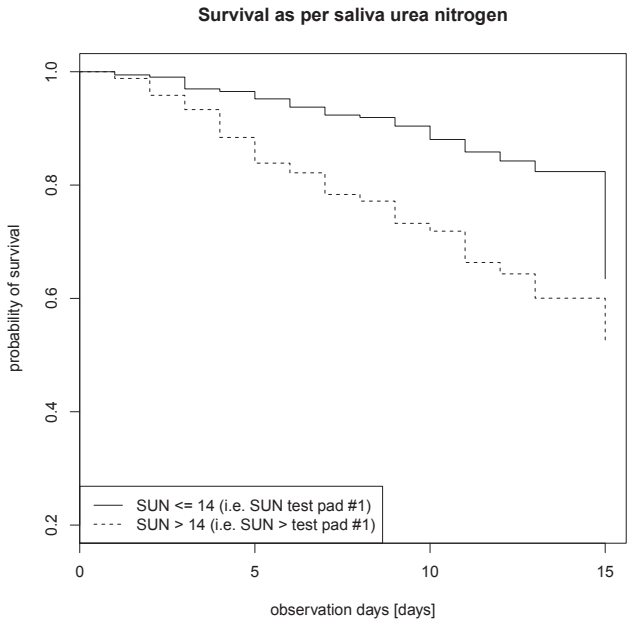

Figure 7: Kaplan-Meier analysis demonstrating survival probability for all patients stratified by the presence or absence of SUN >14mg/dL (i.e. SUN test pad > #1)

DISCUSSION

Simple and inexpensive bedside tools aiding diagnosis and guiding treatment of KD are lacking. The main finding of the present study was that SUN measured by a dipstick demonstrated good diagnostic performance and longitudinal agreement with blood urea nitrogen (BUN) in patients with predominantly acute kidney disease in a LRS in sub-Sahara Africa (SSA).

The results confirm earlier reports on the agreement between SUN and BUN (7, 8). In addition, they corroborate the notion that SUN dipsticks can be used for monitoring the evolution of renal function (15). Of note, BUN and SUN agreed well throughout all observation days. This study also corroborates previous findings of SUN levels being lower than BUN (7, 8, 15). There were no significant differences between the lower and higher test pads of SUN as demonstrated by ANOVA with the post-hoc Bonferroni, which points to the lack of accuracy of SUN to detect more subtle changes in renal function. SUN diagnostic performance decreased over the course of the study period, consistent with previous findings (15).

In this study, SUN > 14mg/dL (SUN > test pad #1) was identified as a threshold to diagnose KD at Day 0 (screening day), with good sensitivity and specificity (0·71 and 0·87 respectively). When assessing the diagnostic performance of SUN levels combined with the information regarding urine output, we found a higher specificity to diagnose KD (0·97) at the expense of lower sensitivity (0·22). These findings demonstrate that SUN strips have a better screening performance (sensitivity) to detect KD when applied alone but a better diagnostic performance (specificity) when used combined with patient reported urine output. This is of significant clinical relevance in our setting. Patients at high risk for AKI (e.g. suffering from malaria or other infectious illness) are routinely evaluated at health centres and district hospitals without access to laboratories (16). A patient, for example, without a change in self-reported urine volume and a SUN < 15mg/dL

(i.e. test pad #1) would have a 3% of chance of having AKI. We believe this is important information that may assist in the care of patients with kidney disease.

Moreover, given the SUN strip's diagnostic performance demonstrated in the present study, and its consistent agreement with BUN on all observation days, our data suggest SUN may be a suitable tool not only to screen for AKI but to also monitor treatment progression in LRS, and should be considered for use at remote health care facilities in patients who are being treated locally for kidney injury. A positive result for SUN should immediately trigger specific diagnostic and therapeutic interventions such as volume assessment and fluid replacement as appropriate (oral or intravenous), removal of nephrotoxins, and management of the underlying disease (e.g. antimicrobials or anti-malarials). A SUN strip suggestive of severe renal injury (e.g. test pads #3-6), or progressive SUN levels over time despite treatment, especially in rural areas with limited healthcare resources, should trigger referral to the next level of healthcare with better infrastructure and possibly renal replacement therapy.

Elevated SUN was demonstrated to be an independent predictor of time to death in this population. This makes SUN an important additional tool in informing clinicians about the severity of a patient's disease process and their prognosis. In LRS this may trigger more intensive monitoring, expedite management and prompt earlier referral to the next tier of the healthcare system with the aim of increasing the probability of survival for these patients.

There are some limitations of the SUN strips to highlight. Limitations for the accuracy of the test and heterogeneity of the results may be related to differences of salivary flow rate and composition in terms of circadian rhythms of salivary glands, variable degrees of patient hydration, and the potential interference of oral bacterial flora with urea degradation in the cavity (17-19). Furthermore, collection of saliva in a patient with reduced conscious level, whilst possible, may be more challenging and was not attempted in this study.

However, there are also major strengths to this technique. The SUN strip is a relatively cheap (approximately US$1/unit), non-invasive, bedside tool of easy application, which allows quick evaluation of renal function. It does not require power or refrigerated storage and may therefore be applicable in even the remotest of settings. In low-middle income countries where many people, due to infrastructural, financial and political issues, often do not receive appropriate diagnosis and treatment (2, 20), SUN strips could be of great utility to increase the identification of renal disease, to improve the diagnostic capability of healthcare facilities, to raise awareness about the need of earlier management and, above all, to improve patient outcome from AKI.

CONCLUSION

This study is the first effort to apply a bedside diagnostic tool to detect and follow-up patients affected by KD in a low resource setting, in this case in sub-Sahara Africa. Here, as in previous studies in developed settings, SUN demonstrated good diagnostic performance to screen, diagnose and follow patients with acute and chronic kidney injury. Moreover, SUN was shown to be an independent predictor of all-cause mortality. Semi-quantitative SUN measurements by a simple dipstick method in parallel with a clinical evaluation may assist in the diagnosis of AKI, especially under circumstances of limited health care resource. Studies will now address the capability of SUN strips to detect KD in different populations and in more rural settings, and assess the impact on patient outcome of this diagnostic tool.

Acknowledgments

We acknowledge the generous provision of SUN dipstick free of charge by Integrated Biomedical Technology. The results presented in this paper have not been published previously.

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Disclosure Statement

Dr. V. Calice-Silva was a fellow of the ISN and received scholarship from the Brazilian Government (CAPES) during part of the time when the study was conducted. Drs. P. Kotanko and N.W. Levin hold stock in Fresenius Medical Care. During this study, RPF received a scholarship from the Brazilian Council for Research Support (CNPq). No other authors have financial interests to declare.

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SUPPLEMENTARY MATERIAL:

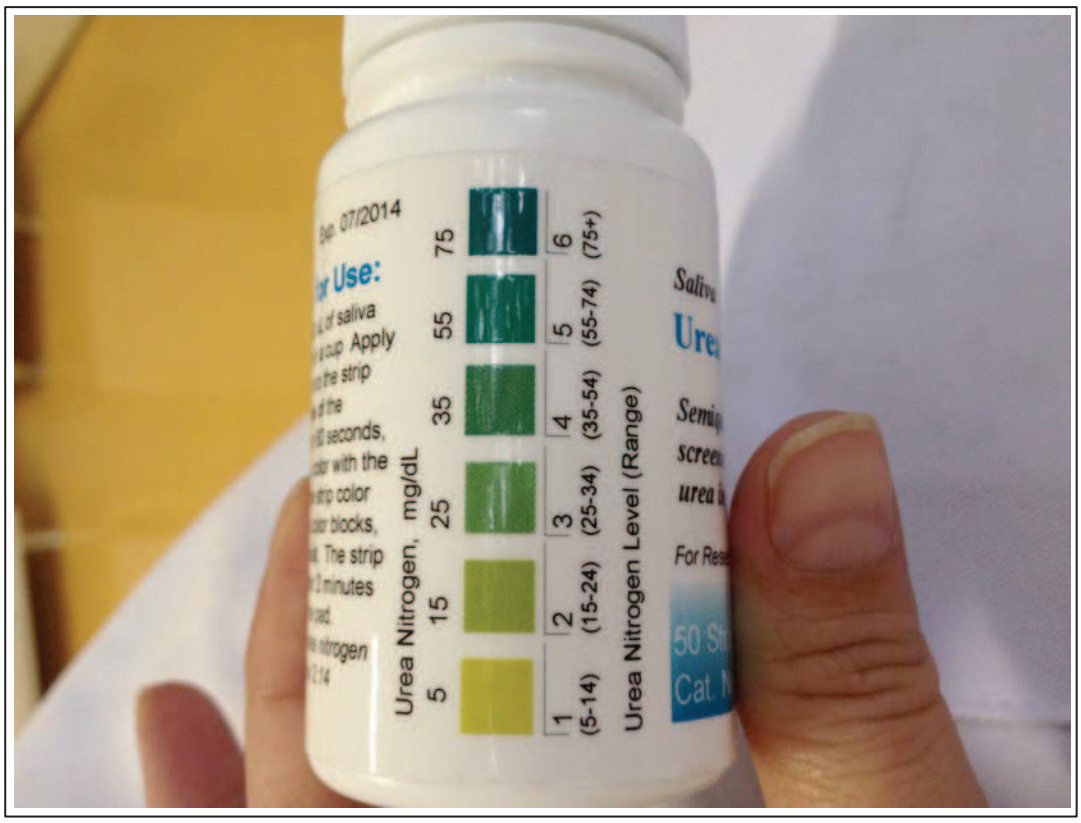
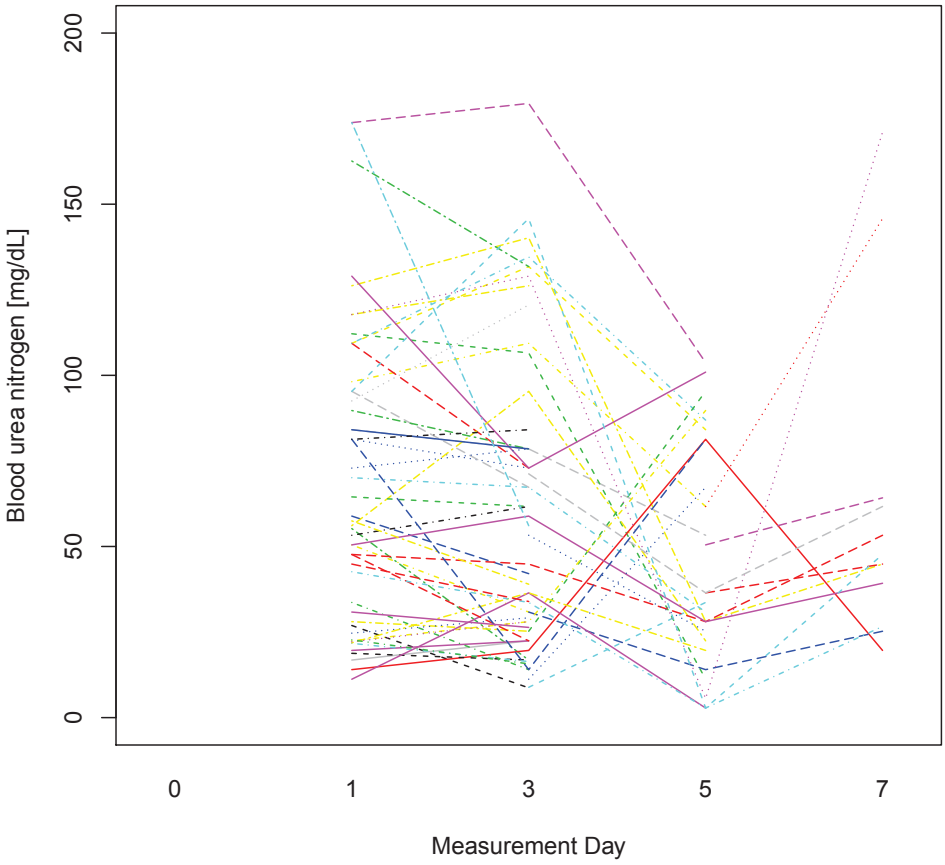


Figure S1: Saliva Urea Nitrogen (SUN) Dipstick reference pads (#1-6) of increasing urea nitrogen range. The change in colour of the test pad is compared to these reference pads.

<table>
<tr><th colspan="3">Confirmed Acute Kidney Injury (AKI)</th></tr>
<tr><td></td><td>Creatinine Criteria*</td><td>Urine Output criteria*</td></tr>
<tr><td>Stage 1</td><td>1·5-1·9 times baseline**/***
OR
≥26·5μmol/l increase****</td><td><0·5ml/kg/h for 6-12 hours</td></tr>
<tr><td>Stage 2</td><td>2·0-2·9 times baseline</td><td><0·5ml/kg/h for ≥12 hours</td></tr>
<tr><td>Stage 3</td><td>3·0 times baseline
OR
Increase in creatinine to ≥353·6μmol/l*****
OR
Initiation of RRT</td><td>< 0·3ml/kg/h for ≥24 hours
OR
Anuria for ≥12 hours</td></tr>
<tr><td colspan="3">*basis of diagnosis can be with either creatinine criteria or urine output criteria, with most advanced stage on either determining maximum stage
**baseline creatinine = lowest creatinine within last year; if not known, creatinine baseline estimated assuming eGFR of 75ml/min per 1·73 m2 (via MDRD equation), or taken as lowest creatinine during hospital stay (whichever lower)
***known or assumed to have occurred within last 7 days
****within 48 hours
*****must also fulfill at least stage 1 criteria</td></tr>
<tr><th colspan="3">Acute Kidney Disease/Disorder (AKD) without AKI*</th></tr>
<tr><td colspan="3">GFR <60 ml/min per 1·73 m^2 for <3 months**
OR
Decrease in GFR by ≥35% or increase in SCr by >50% for <3 months**</td></tr>
<tr><td colspan="3">*not fulfilling AKI criteria
**no biochemical (previous creatinine) or structural (kidneys <9cm bilaterally) evidence kidney damage >3 months old</td></tr>
<tr><th colspan="3">Chronic Kidney Disease (CKD)</th></tr>
<tr><td colspan="3">GFR <60 ml/min per 1·73 m^2 for >3 months*</td></tr>
<tr><td colspan="3">*biochemical (previous creatinine >3 months old) or structural (kidneys <9cm bilaterally) kidney damage >3 months old</td></tr>
<tr><th colspan="3">No Kidney Disease (NKD)</th></tr>
<tr><td colspan="3">GFR by ≥60ml/min per 1·73 m^2, stable serum creatinine, and no evidence structural kidney damage >3 months old*</td></tr>
<tr><td colspan="3">*includes patients with serum creatinine within reference range at screening, and patients with creatinine outside reference range at screening but who did not subsequently fulfill definition of AKI, AKD or CKD</td></tr>
</table>

Figure S2: Definitions of AKI, AKD without AKI, CKD and NKD. Kidney Disease (KD) includes AKI, AKD without AKI, and stable CKD. AKD incorporates both AKI and AKD without AKI.


Blood urea nitrogen [mg/dL]
0
50
100
150
200
0
1
3
5
7
Measurement Day

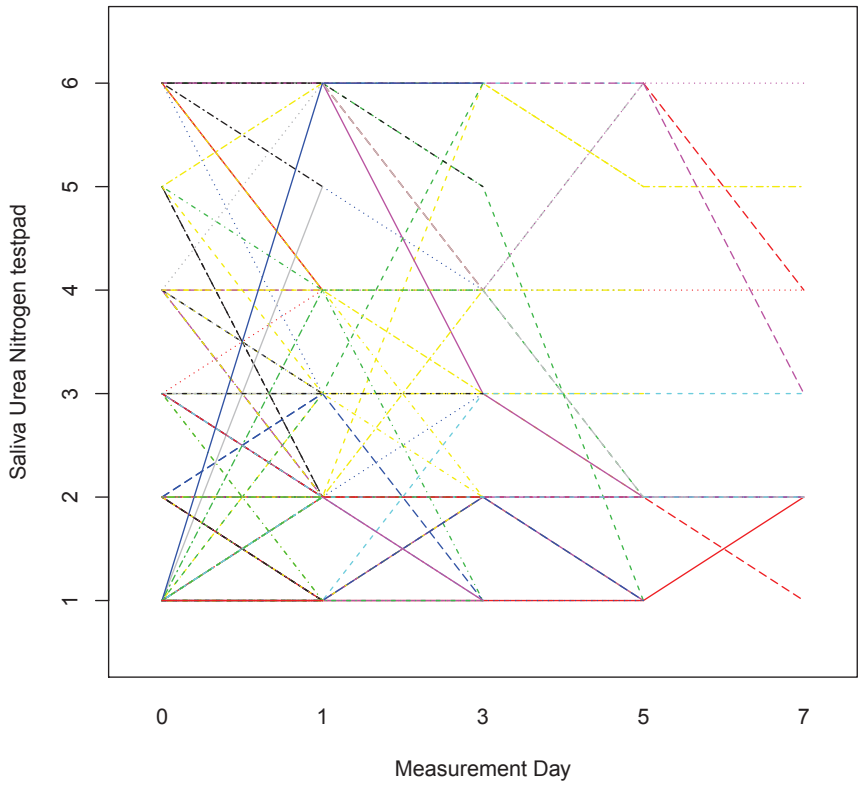


Figure S2: Line plots representing a) BUN and b) SUN at all observation days

Table S1: Demographic and clinical-related parameters on day 1 in patients with kidney dysfunction. The volume status (hyper/eu/hypovolaemic) was assessed by the clinical study team. Urine output and thirst are based on patient reports.

	N volume status assessed [count]	Hypervolemic [%]	Euvolemic [%]	Hypovolemic [%]	N urine assessed [count]	Less urine [%]	Normal urine [%]	No urine [%]	No answer urine [%]	Thirst assessed [count]	More thirst [%]	No answer thirst [%]
No kidney disease	21	23·81	42·86	33·33	21	19·05	66·67	0	14·29	12	75	25
AKI stage 1	21	9·52	52·38	38·1	23	26·09	69·57	0	4·35	16	81·25	18·75
AKI stage 2	17	5·88	47·06	47·06	17	17·65	76·47	0	5·88	11	90·91	9·09
AKI stage 3	43	20·93	18·6	60·47	44	29·55	70·45	0	0	23	100	0
AKD with no AKI	20	30	30	40	20	25	70	0	5	15	93·33	6·67
CKD	5	40	60	0	5	20	80	0	0	4	100	0
All	127	19·69	35·43	44·88	130	24·62	70·77	0	4·62	81	90·12	9·88

AKI: Acute kidney injury – KDIGO stages 1, 2 and 3
AKD without AKI: Acute kidney disease/disorder without AKI
CKD: Chronic Kidney disease

SUN (mg/dL)	SUN test pad	Tests count	Median BUN [mg/dL]	BUN [mg/dL] 25th percentile	BUN [mg/dL] 75th percentile
5 to 14	1	63	22·30	16·12	35·74
15 to 24	2	86	33·64	22·47	58·46
25 to 34	3	29	64·48	47·66	75·70
35 to 54	4	27	67·29	47·66	113·55
55 to 74	5	8	84·11	63·08	95·33
above 75	6	36	106·54	80·60	135·98

Table S2: BUN levels of all patients on all observation days stratified as per SUN. ANOVA with post-hoc Bonferroni correction was employed to compare BUN stratified by measured SUN categories and showed significant differences between test pad 1# and test pads #3, #4, #5 and #6, between test pads #2, #3 and #4 and test pad #6 and between test pad #2 and test pad #4. A lack of significant differences between BUN and SUN concentrations at lower SUN ranges (i.e. test pads #1 versus #2 and #2 versus #3) was observed.

5 – Final considerations

This thesis is the result of a international collaborative project developed through a partnership between the Pontifícia Universidade Católica do Paraná, Curitiba, and the Renal Research Institute (RRI), New York, USA, which started in 2011. Through this partnership the idea to study SUN strips in AKI patients was initiated.

The SUN project represented a great opportunity of networking and teamwork. Once SUN dipstick is a tool, which could be of great importance in disadvantages areas we could work on collaborative projects through the ISN research and prevention program and extend the execution of our project to other areas worldwide where we believe this tool could be very helpful, such as some countries in Africa (Malawi and Angola).

With this project we were able to study the diagnostic performance of SUN as a bedside tool to detect AKI. We believe the strips could potentially improve the diagnostic capabilities of health care centers at low-resources setting to detect renal disease, especially AKI. With the first we demonstrated that SUN strips have a similar diagnostic performance of BUN, especially to detect patients at the worst degree of AKI (AKIN 3). In the second study again SUN showed a good agreement with BUN at AKIN stage 3, which was consistent at all the observational days. These findings may indicate SUN dipstick as a good tool to detect and monitoring patients with AKI along the time.

However, the setting where these two studies were performed is not considered low-resources, where the tool in study would be of greater value.

Because of that the third study was proposed. Therefore, SUN strips were applied at a general hospital in Malawi and demonstrated to be a feasible tool to identify AKI. The previous findings of the agreement between BUN and SUN over the follow-up days were confirmed, and also a similar sensibility and specificity of SUN to detect all stages of AKI (72% and 87% compared to 91% and 79% to AKIN 3 – study 1) was observed. Moving one step forward, clinical parameters were added to the model, namely urine output (UO) and thirst self-reported for the patients. The parallel analyses demonstrated even higher specificity for SUN and UO associated (97%). Which means that if a patient arrives at a remote health care center for whatever reason, and when asked he refers normal urine output and present a SUN strip test negative there is a 97% chance that this patients does not have AKI.

Therefore, SUN dipstick is a promising tool to be used at the bedside in patients with suspected renal disease in lower resource areas. However, there are some limitations, which need to be addressed to improve this tool and make it more accurate particularly in the earlier staged of AKI. In our hands SUN had a good diagnostic performance to detect levels of BUN higher than 50 mg/dl, but was not efficient to detect small changes on that, increasing the risk of to misdiagnosis patients with mild and moderate AKI. One reason for that finding could be explained for changes in the salivary pH in patients suffering from AKI, also the interference of some specific bacterial flora producing urease inside the mouth causing an underestimation of SUN[47, 55]. Medications effect, which could change salivary glands function leading to dry mouth (xerostomy) which is

very common in patients with renal dysfunction [38, 56-58]. The manufacture of the SUN strip test is aware of these limitations and is working on strategies to minimize these issues.

Other important factor has to be addressed is how to deal with the interference of the bacterial flora present inside the mouth and with the circadian rhythms of salivary glands. A good strategy could be the association of oral hygiene evaluation to patient's clinical evaluation. Finally, regarding the circadian rhythm of SUN, avoidance to measure BUN and SUN in different periods of the day may minimize these variations.

However, besides all the limitations of SUN mentioned above, the strips had similar results in all the three studies, which were performed at completely different populations in terms of socioeconomic, cultural and health care aspects. This makes SUN dipsticks a reliable tool to be used with the purpose it has been proposed.

Further improvements of the diagnostic tool are ongoing, and this may allow the increase in sensibility and specificity to detect low BUN levels, improving SUN capability to detect AKI at lower stages of the disease which may help to the early identification of patients with AKI and allowing the proper treatment, and stall the progression of the disease to later stages, which is extremely important considering the scarce resources to renal replacement therapy in developing countries.

Finally, further studies are needed to evaluate the capability of this tool to diagnose renal impairment in different populations, such as children, pregnant

women and others. As a proof of principle, we have been working on studies in children to replicate the evaluation of SUN as performed in the adult population. Another study is in the planning stage in the obstetric population, children with AKI and in patients with malaria in Africa.

The potential applicability of this tool in the future would be in the screening of patients under risk of renal disease living in remote areas without access to laboratory facilities, to evaluate the response to the treatment and guide the physician or health care worker's decision to transfer the patient to a more structured health care center. It could be also used in situations of major disasters. All this applications should be aligned with global healthcare strategies and with the initiative of global organization, such as the 0 by 25 initiative from the International Society of Nephrology. The group involved in the present studies is aligned with ISN's projects and vision, and we hope our work provides a contribution to the ambitious and very relevant projection of reducing preventable deaths due to AKI in the developing world.

6 – Conclusion

According the results presented in this thesis, SUN has an equivalent diagnostic performance compared to BUN to detect acute kidney injury, especially at stage 3 (KDIGO/AKIN). In the follow up study, the agreement between BUN and SUN was comparable on all observation days, generally following a declining trend in BUN. Even though SUN strips underestimate BUN, SUN reliably reflects BUN and follows its changes over time, particularly at higher levels of BUN (>50mg/dL). SUN combined with subjective information regarding urine output presents even higher specificity at all stages of AKI. Therefore, SUN testing associated with a thorough clinical evaluation may assist in the identification of patients with suspected AKI, especially under circumstances of limited health care resources.

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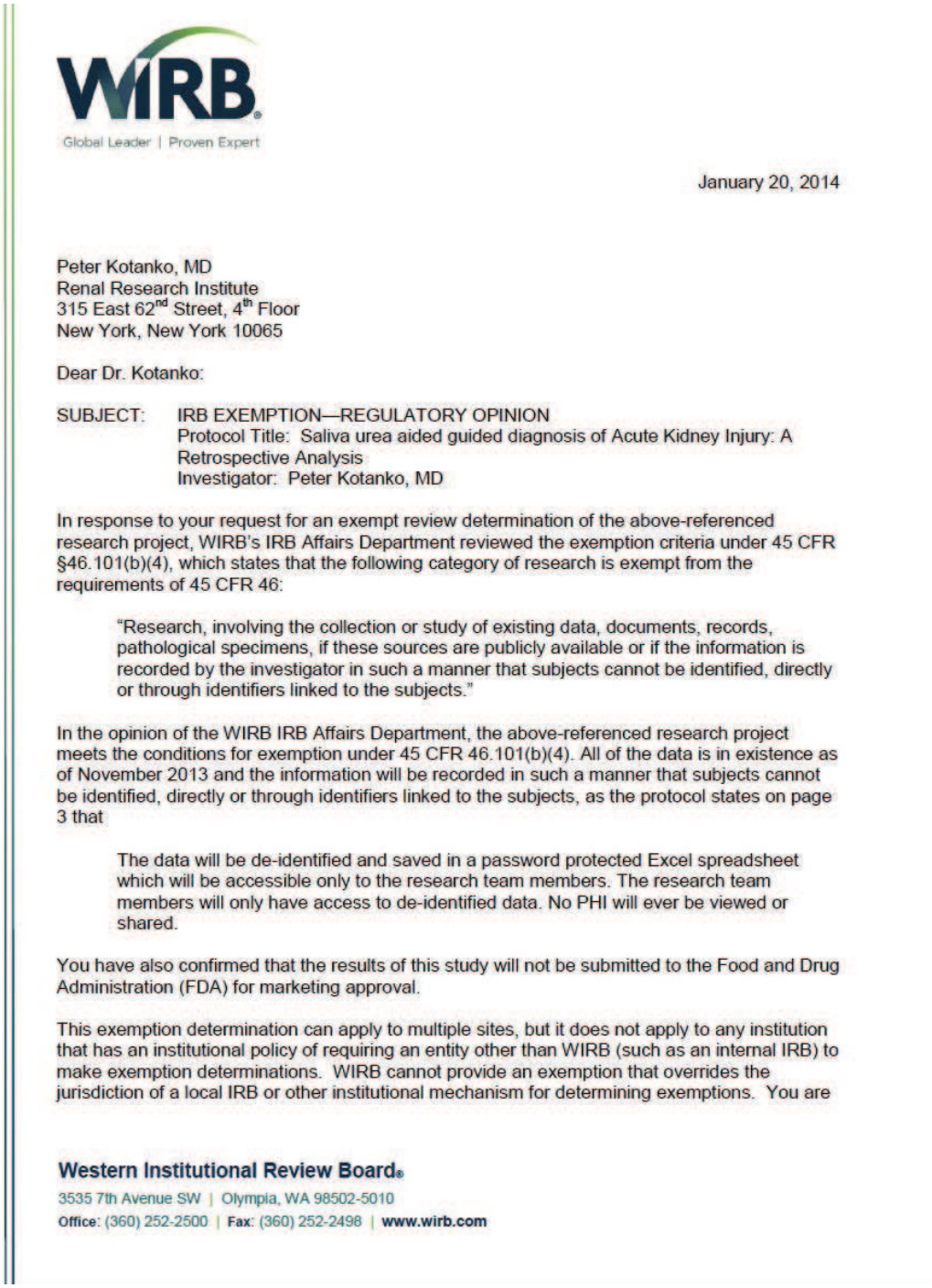
8 – Appendix

8.1 – Letter of approval IRB



WIRB
Global Leader | Proven Expert

January 20, 2014

Peter Kotanko, MD
Renal Research Institute
315 East 62nd Street, 4th Floor
New York, New York 10065

Dear Dr. Kotanko:

SUBJECT: IRB EXEMPTION—REGULATORY OPINION
Protocol Title: Saliva urea aided guided diagnosis of Acute Kidney Injury: A Retrospective Analysis
Investigator: Peter Kotanko, MD

In response to your request for an exempt review determination of the above-referenced research project, WIRB's IRB Affairs Department reviewed the exemption criteria under 45 CFR §46.101(b)(4), which states that the following category of research is exempt from the requirements of 45 CFR 46:

> "Research, involving the collection or study of existing data, documents, records, pathological specimens, if these sources are publicly available or if the information is recorded by the investigator in such a manner that subjects cannot be identified, directly or through identifiers linked to the subjects."

In the opinion of the WIRB IRB Affairs Department, the above-referenced research project meets the conditions for exemption under 45 CFR 46.101(b)(4). All of the data is in existence as of November 2013 and the information will be recorded in such a manner that subjects cannot be identified, directly or through identifiers linked to the subjects, as the protocol states on page 3 that

> The data will be de-identified and saved in a password protected Excel spreadsheet which will be accessible only to the research team members. The research team members will only have access to de-identified data. No PHI will ever be viewed or shared.

You have also confirmed that the results of this study will not be submitted to the Food and Drug Administration (FDA) for marketing approval.

This exemption determination can apply to multiple sites, but it does not apply to any institution that has an institutional policy of requiring an entity other than WIRB (such as an internal IRB) to make exemption determinations. WIRB cannot provide an exemption that overrides the jurisdiction of a local IRB or other institutional mechanism for determining exemptions. You are

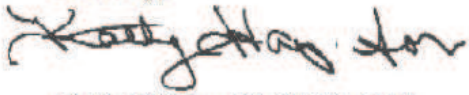
Western Institutional Review Board®
3535 7th Avenue SW | Olympia, WA 98502-5010
Office: (360) 252-2500 | Fax: (360) 252-2498 | www.wirb.com

responsible for ensuring that each site to which this exemption applies can and will accept WIRB's exemption decision.

WIRB does not impose an expiration date on its IRB exemption determinations. Please note that any future changes to the project may affect its exempt status, and you may want to contact WIRB about the effect these changes may have on the exemption status before implementing them.

If you have any questions, or if we can be of further assistance, please contact Paul Newton, J.D., C.I.P., at 360-252-2422, or e-mail RegulatoryAffairs@wirb.com.

Sincerely,



R. Bert Wilkins, J.D., M.H.A., C.I.P.
Executive IRB Chair

RBW:PN:hnb
B4-Exemption-Kotanko (01-20-2014)
cc: WIRB Accounting
WIRB Work Order #1-819655-1

This document electronically reviewed and approved by Harp, Kathy on 1/20/2014 10:34:29 AM PST. For more information call Client Services at 1-360-252-2500.

CERTIFICATE OF ETHICS APPROVAL

This is to certify that the College of Medicine Research and Ethics Committee (COMREC) has reviewed and approved a study entitled:

P.11/14/1660– A study of Acute Kidney injury in Malawi : Incidence , Aetiology outcomes and the use of a Salivary Urea Nitrogen Dipstick version 3 dated February 2015 by Dr. Rhys Evans

On 16th March 2015

As you proceed with the implementation of your study, we would like you to adhere to international ethical guidelines, national guidelines and all requirements by COMREC as indicated on the next page

Approved by College of Medicine
16 MAR 2015
(COMREC)
Research and Ethics Committee

Dr. C. Dzamalala– Vice Chairperson (COMREC)

16th March, 2015
Date

8.2 - Parallel production during the PhD period

8.2.1 - Book Chapter

In press:

1. **CALICE-SILVA**, V.; LEVIN, N. et al. Saliva Urea Nitrogen as a biomarker of kidney disease. In: Biomarkers in Disease: Methods, Discoveries and Applications: Kidney. Ed. Springer.

8.2.2 - Published manuscripts

1. **CALICE-SILVA, V.;** PECOITS-FILHO, Roberto et al. Associations Between Global Population Health Indicators and Dialysis Variables in the Monitoring Dialysis Outcomes (MONDO) Consortium. Blood Purif 2015; 39:125-136. doi: 10.1159/000368980.

2 - **CALICE-SILVA, V.;** PECOITS-FILHO, Roberto et al. Saliva urea nitrogen dipstick - a novel bedside diagnostic tool for acute kidney injury. Clin Nephrol. 2014 Dec; 82(12): 358-66. doi: 10.5414/CN108370.

3 - HAN, M.; WILLIAMS, S.; MENDONZA, M.; YE, X.; ZHANG, H.; **CALICE-SILVA, V.**; THIJSSEN, S.; KOTANKO, P.; MEYRING-WOSTEN, A. Quantifying Physical Activity Levels and Sleep in Hemodialysis Patients Using a Commercially-Available Activity Tracker – in press Blood Purification - DOI: 10.1159/000441314.

8.2.3 - Accepted manuscripts

1 – RAIMANN, J.; CALICE-SILVA, V.; PECOITS-FILHO, R. et al. Saliva Urea Nitrogen Continuously Reflects Blood Urea Nitrogen after Acute Kidney Injury Diagnosis and Management: Longitudinal Observational Data from a

Collaborative, International, Prospective, Multicenter Study – in press Blood Purification - DOI: 10.1159/000445041.

8.2.4 – Abstracts presented at international meetings

1- **CALICE-SILVA, V.**; PECOITS-FILHO, R. et al. Predictors of Sudden Cardiac Death in Hemodialysis Patients with and without Previous Arrhythmia– Results From a Multinational Cohort In: Kidney Week, 2015, San Diego, CA. American Society Nephrology.

2- **CALICE-SILVA, V.**; PECOITS-FILHO, R. et al. Dynamics of nutritional and metabolic markers before death in peritoneal dialysis: results from BRAZPD II, a nationwide prospective study. 52nd European Renal Association – European Dialysis and Transplantation Association Congress, London, UK.

3 – **CALICE-SILVA, V.**; PECOITS-FILHO, R. et al. Associations between anemia parameters and global health indicators in dialysis patients: results from the MONDO initiative. In: World Congress of Nephrology, 2015, Cape Town, South Africa. International Society of Nephrology.

4 - **CALICE-SILVA, V.**; PECOITS-FILHO, R. et al. Multinational study of the relationship between fluid status and left ventricular structure and function in chronic hemodialysis patients. In: World Congress of Nephrology, 2015, Cape Town, South Africa. International Society of Nephrology.

5 - **CALICE-SILVA, V.**; PECOITS-FILHO, R. et al. Predictors of sudden death in incident patients on hemodialysis – results from the international MONDO Initiative. In: Kidney Week, 2014, Philadelphia, PA. American Society Nephrology.

6 - **CALICE-SILVA, V.**; PECOITS-FILHO, R. et al. Predictors of Arrhythmia events in incident patients on hemodialysis – results from the International MONDO Initiative. In: Kidney Week, 2014, Philadelphia, PA. American Society Nephrology.

7 – **CALICE-SILVA, V.**; PECOITS-FILHO, R. et al. Predictors of Congestive Heart Failure Events in Incident Patients on Hemodialysis – Results From the International MONDO Initiative. 51st European Renal Association – European Dialysis and Transplantation Association Congress, Amsterdam, 2014.

8- **CALICE-SILVA, V.**; PECOITS-FILHO, R. et al. Saliva Urea Nitrogen continuously reflects BUN after AKI diagnosis and management: a prospective longitudinal study. 51st European Renal Association – European Dialysis and Transplantation Association Congress, Amsterdam, 2014.

9 - **CALICE-SILVA, V.**; PECOITS-FILHO, R. et al. Spit out to check your kidneys: saliva urea nitrogen dipstick - a novel bedside diagnostic tool for acute kidney injury. In: Kidney Week, 2013, Atlanta. American Society Nephrology Week, 2013.

8.2.5 - Awards

- **2015:**
    - ISN Fellow's Poste Award – World Congress of Nephrology, Cape Town, South Africa.
        - Abstract: Multinational study of the relationship between fluid status and left ventricular structure and function in chronic hemodialysis patients
- **2014:**

- ISN grant – Clinical Research and Prevention Program
    - Project: Salivary Urea Nitrogen as a diagnostic tool in Acute Kidney Injury.
- Renal Therapy Group Excellence Award by the Renal Research Institute, New York, USA.
- One of the 8 best young nephrologist investigators abstracts by the European Renal Association- European Dialysis and Transplantation Association in the 51st Congress, Amsterdam.
    - Abstract: Predictors of Congestive Heart Failure Events in Incident Patients on Hemodialysis – Results From the International MONDO Initiative.
- Travel grant from ERA-EDTA to the 51st Congress, Amsterdam.

- **2013:**
    - Scholarship from CAPES, Brazil to spend one year at RRI, New York, USA through the Sandwich PhD program.
    - ISN fellowship at RRI, NY, USA – from May to July.

GUARANTEE OF PRIZE

FELLOWSHIP AWARD 2015

Dear Dr. Silva,

Thank you very much for participating to the ISN Programs Poster Session, it was a pleasure to meet you today.

On behalf of the **Fellowship Committee**, I would like to congratulate you on being the winner of the **Fellowship 3rd Prize** at the World Congress of Nephrology 2015 (Cape Town).

We would like to remind you to be present at the **Plenary Session 6, on Monday 16th March at 9.45am in the Hall 4A/B for the ISN Poster Awards Ceremony**. John Feehally, ISN Programs Chair will present the ISN Programs Awards.

The ISN herewith confirms that the monetary award of **500 USD** will be transferred to your personal bank account after the Congress.

Please find here enclosed the requested documents to submit to us after the Congress.

Thank you for sending us a faxed (fax number is +32 2 808 44 54) or a scanned copy of the forms by e-mail to Christine Rugurika (CRugurika@theisn.org)

Kind regards,

ISN Programs Team

International Society of Nephrology (ISN)

Global Operations Center

Rue des Fabriques 1 | B-1000 Brussels | Belgium

Main: + 32 2 808 04 20| Fax +32 2 808 44 54

ISN

International Society of Nephrology

www.theisn.org

Global Operations
Rue des Fabriques 1
B-1000 Brussels – Belgium
Tel: +32 2 808 04 20
Fax: +32 2 808 44 54
Email: info@theisn.org

US Operations
340 North Avenue 3rd Floor
Cranford, NJ 07016-2496
United States of America
Tel: +1 567 248 9703
Fax: +1 908 272 7101
Email: info@theisn.org

August 3, 2015

Dear **Dr. Pecoits-Filho**
Dear **Dr. Calice da Silva,**

The Selection Committee of **ISN Clinical & Research Program** has provided us with the scores and final recommendation for funding the projects submitted to **Round 2 of the 2014 Call** for Proposals. In this Round we received **14** applications. As in the past, the proposals have been considered for funding according to the ranking of the average scores of the **ISN Clinical & Research Program Committee** members

I am very pleased to announce that your proposal **"SALIVARY UREA NITROGEN AS A DIAGNOSTIC TOOL IN ACUTE KIDNEY INJURY"** has been awarded with **15,000 USD.**
The above-mentioned proposal is being managed by Dr. Roberto Pecoits-Filho, the institutional coordinator and by Viviane Calice da Silva, the study coordinator.

Thank you again for participating to our ISN CLINICAL RESEARCH PROGRAM

Regards,

Cello Tonelli
Chair, ISN Clinical Research Committee

ISN
International Society of Nephrology
www.theisn.org

Global Operations
Rue des Fabriques 1
B-1000 Brussels – Belgium
Tel: +32 2 808 04 20
Fax: +32 2 808 44 54
Email: info@theisn.org

US Operations
340 North Avenue 3rd Floor
Cranford, NJ 07016-2496
United States of America
Tel: +1 567 248 9703
Fax: +1 908 272 7101
Email: info@theisn.org

ERA-EDTA 51st Congress - Travel Grant

De: **ERA-EDTA abstracts** (abstracts@era-edta.org)
Enviada: terça-feira, 1 de abril de 2014 16:15:07
Para: vivicalice@hotmail.com

Dear Dr Silva,

We are pleased to inform you that you have won one of the 2014 ERA-EDTA Travel Grants of EUR 1000 and a complimentary registration to attend the 51st ERA-EDTA Congress, organised in Amsterdam, the Netherlands (May 31-June 3, 2014). This decision is based on the evaluation of your abstract **"PREDICTORS OF CONGESTIVE HEART FAILURE EVENTS IN INCIDENT PATIENTS ON HEMODIALYSIS - RESULTS FROM THE INTERNATIONAL MONDO INITIATIVE"**.

You will soon be contacted by the ERA-EDTA Administration Office in order to arrange the payment.

At the Congress, you will receive a diploma and your grant directly at the ERA-EDTA Congress Office desk, located in the Registration Area of the Amsterdam RAI, after the end of your poster presentation.

Travel Grants will be given only to the presenting author winner of the grant, who must show a valid identification card to the ERA-EDTA Congress Office staff.
Awards **will not be given** to co-authors/colleagues/friends/relatives; no exceptions will be made.

As a winner of one of the 2014 ERA-EDTA Travel Grants, you will be entitled to receive registration to the congress free of charge. Your registration confirmation letter will be sent to you shortly; if you have already paid your pre-registration then you will be reimbursed after the Congress. Your badge will be mailed to your address. Your congress bag and other congress materials are to be collected at the bag desks located on the ground floor of the Amsterdam RAI starting May 30, 2014 from 11.00 to 19.00.

Looking forward to seeing you in Amsterdam and congratulations.

Yours sincerely,

Denis Fouque
Chair, Paper Selection Committee

Coordenação de Aperfeiçoamento de Pessoal de Nível Superior
SBN, Quadra 02, lote 06, Bloco L
70.040-020 - Brasília, DF
Brasil

C A P E S

December 27, 2013

TO WHOM IT MAY CONCERN

We hereby certify that Mr./Mrs. **VIVIANE CALICE DA SILVA** has been awarded a scholarship from the CAPES Foundation, an agency under the Ministry of Education of Brazil, in order to conduct part of his doctoral research as a Visiting Graduate Student at **RENAL RESEARCH INSTITUTE**.

The scholarship includes:

. up to 12 monthly stipends of US$ 1.300,00;
. a one-time settling-in allowance of US$ 1300,00;
. a health insurance allowance, paid directly to the grantee, of US$ 1080,00 /concession period;
. displacement allowance towards the cost of travel for the itinerary below:
CURITIBA(BR)/NOVA IORQUE(EU)/CURITIBA(BR);

The scholarship is valid from **March/2014** to **February/2015**.

Sincerely,

LUIS FILIPE DE MIRANDA GROCHOCKI
General Coordinator of Scholarships Abroad
This document is public and does not need signature recognition - Article 19, interpolated proposition II, Federal Constitution of Brazil.

Luis Filipe de Miranda Grochocki
Coordenador Geral de Bolsas e Projetos
Port. nº 1.205 – DOU – 18/12/2013
CAPES/MEC

International Society of Nephrology

ISN Fellowship Certificate

Hereby, I, Fellowship chairman of the International Society of Nephrology, declare that

Dr. Viviane Silva

from Brazil has successfully completed her ISN Fellowship.
She was trained at "Renal Research Institute" USA under the supervision of Professor Peter Kontanko, between May 2013 and July 2013.

David Harris

Professor David Harris