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Pre and post analytical pitfalls of neutrophil gelatinase-associated lipocalin and cell cycle arrest biomarkers predicting acute kidney injury

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ABSTRACT

AKI disorder is one of most serious clinical conditions recognized worldwide. In recent years, many improvements were made both for renal biomarkers and to know better the total testing process (TTP) in laboratory medicine including pre and post analytical phases. However, TTP for new renal biomarkers remains an issue. We conducted the present study to determine pre/post-analytical pitfalls of neutrophil gelatinase-associated lipocalin (NGAL) and tissue inhibitor of metalloproteinase 2-insulin-like growth factor binding protein 7 [TIMP-2]-[IGFBP-7] called Nephrocheck test. Material and Methods. We analyzed urinary samples collected in two previous studies at intensive care unit (ICU) from an adult population undergoing cardiac surgery. We performed measurements using a chemiluminescent method on an automatic analytical platform and a point of care testing Nephrocheck (Astute-Meter). Biochemical results were analyzed without and with the urine creatinine (uCrea) correction. Furthermore, a method to calculate the mean variation rate was applied for different biomarker concentrations using differential equations. Results. A received operating curve for predicting AKI was obtained with AUC value= 0.66 corresponding to the better NGAL cut-off= 29 ng/mL (p= 0.016). A significant association between the NGAL increase and AKI severity was achieved (p= 0.02). NGAL increases showed: NGAL pre= 18.3 (7.7-23.7) ng/mL, NGAL post= 19.2 (3.8-86) ng/mL median (interquartile range) statistically not significant. Analyzing the corresponded urinary creatinines, we found many uCreas with very low concentrations suggesting a possible dilution of patient samples collected. Correction with the urinary creatinine did not have added significant outcomes. It seemed that [TIMP-2]-[IGFBP-7] expressed as Nephrocheck AKI score $(ng/mL)^2/1000$ introduces a variation due to the amplification of TTP errors. Model of differential equations applied to Nephrocheck test results demonstrated a mean variation rate that rises as the biomarker concentration increases. Conclusions: NGAL results on urinary testing had a modest diagnostic accuracy probably due to pre-analytical variability in ICU critical patients. Pre and Post-analytical variability affected Nephrocheck results as observed by the expression of measurements $(ng/mL)^2/1000$. In addition, the too low numerical measurements as fraction of 1 and high variability around the AKI risk score cut-off= 0,3 might determine possible overlap between different patient groups. We suggested an analytical expression for the cell cycle arrest biomarkers as cumulative concentration in linear form (ng/mL).

Keywords: *Pre/post analytical variability, NGAL, Nephrocheck AKI score, acute kidney injury, differential equation*

1. Introduction

Acute kidney injury (AKI) is a common complication in several diseases as well as hematologic and no hematologic cancer, infection diseases, cardiovascular diseases especially following cardiac surgery. Laboratory diagnostics is more and more a growing field with decisive contribution to modern medicine by supporting the prevention, diagnosis and therapeutic monitoring of human disorders [1]. It is now well established that pre-analytical phase affects the stability of human biological tissue (urine or plasma) with consequences for both basic proteomic experiments and biomedical testing [1,2,3,4].

Furthermore, in laboratory medicine it is important to consider the complete diagnostic process called total testing process (TTP) including the post-analytical phase too. Actually, standard procedures and diagnostic common

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protocols for pre and post-analytical phase applied accurately to clinical practice remain an issue. Biomedical research suggests that many so-called "biomarker candidates" fail to progress beyond the discovery phase, and much emphasis is placed on pre and post-analytical variability [3]. In our diagnostic assay experience, we though that better understanding of the overall process from the biomarker discovery to assay validation are need. In fact, pre -analytical phase should be added to the diagnostic appropriateness of clinical testing, in instance for diagnosis or early diagnosis. To date, the diagnostic tools on the acute kidney injury (AKI) diagnosis have been disappointing, even if new biomarkers are extensively suggested [5,6], as well as, the neutrophil gelatinase-associated lipocalin (NGAL) and particularly the cell cycle arrest proteins: tissue inhibitor of metalloproteinase 2-insulin-like growth factor binding protein 7 (TIMP-2)-(IGFBP-7). Combination of the two proteins was called Nephrocheck test performing measurements in urinary samples. Both biomarkers are frequently recognized as useful for AKI detection after cardiac surgery in a timely manner to prevent adverse outcomes in clinical practice. In fact, Nephrocheck test was considered superior in early AKI detection in critically ill patients. However, diagnostic tests are affected by different biological and analytical factors producing a significant variability and uncertainty. Despite a huge of literature supported the new biomarkers for assessing AKI risk, many doubts remain in regard of their applicability in clinical practice. We conducted the present study to determine diagnostic pitfalls of NGAL and [TIMP-2]-[IGFBP-7] expressed as Nephrocheck score in the early AKI diagnosis after cardiac surgery (CSA-AKI). We focused on pre-analytical phase as sample collection and sampling time then on post-analytical phase as result expression. Hence, evaluating experimental data, the investigation concerning pre and post-analytical features of renal biomarkers, aimed to detect the causes of our diagnostic outcomes in urinary samples belonging to an intensive care unit (ICU) patient population.

2. Material and Methods

A subset of thirty-two patients (females=nine, males= twenty-three) with complete data, belonging to a previous study on the CSA-AKI [see reference 7] was analyzed for urinary NGAL (uNGAL) measurements (ARCHITECT i1000SR® analyzer, Abbott Diagnostics GmbH, Wiesbaden, Germany) to evaluate the diagnostic accuracy parameters: sensitivity, specificity, received operating characteristic (ROC) curve. Patients were undergoing cardiac and cardiovascular surgery, provided a written consensus as stated by the local Ethic Committee. Urinary samples were collected in ICU according to the collection procedure of manufacturer. Patients were selected following the AKI risk inclusion criteria with two or more conditions: 1) age > 70 years, 2) eGFR < 60mL/min/1.73m2 (estimated by MDRD formula), 3) ejection fraction (EF) < 41%, 4) redo operation, 5) combined surgery. Consecutively, we performed a similar study, always through the same conditions and working team (laboratorians and clinicians), measuring the [TIMP-2] -[IGFBP-7] Nephrocheck score (Astute Medical, San Diego, CA, USA) on sixty-eight patients [see reference 8]. An expert clinician team in ICU following the KDIGO criteria formulated the AKI diagnosis. For the AKI definition, all the renal injury stages were considered equally, from AKI stage 1 (low severity) to AKI stage 3 (high severity). Sampling time was the same for the two studies: before surgery (baseline, pre-biomarker) and within 4 hours from the patient arrival in ICU (post-biomarker), to obtain an early detection of kidney impairment. We performed data analysis considering urinary creatinine (uCrea) as dilution marker and the expression of AKI biomarkers corrected by its concentration. A critical evaluation of experimental data in regard of pre and post-analytical variability was made using new methodological tools: in particular interpreting consecutive results for the uNGAL and applying a mathematical model for the Nephrocheck test. Statistics have been carried out on continuous variables using the t-test for independent samples. Variables not normally distributed were presented as median and interquartile ranges and compared with the Wilcoxon rank-sum test. Categorical data were compared using the chi-square test or the Fisher exact test, as appropriate. Receiver operating characteristic (ROC) curves were calculated and the area under curve (AUC) with 95% confidence interval (CI) was used to measure the NGAL prediction for AKI. Analysis were performed by SAS version 9.4 (SAS Institute Inc., Cary, NC). Correlation study was performed through Pearson correlation with a statistical significance of 0,05.

We also evaluated the uNGAL increases (pre-post surgery), considering a clinically significant increase in urinary samples, uNGAL (positive test), if the second value post-surgery was ≥50 ng/mL [7,9]. Post-analytical phase was investigated focusing on the expression of biological measurements, in particular the [TIMP-2]-[IGFBP-7] measure, Nephrocheck AKI score expressed as (ng/mL) 2/1000. We applied a mathematical model (differential equation) to estimate the variation rate of the assay [10] (see below).

Furthermore, we simulated a systematic error by dilution of the samples: 1 control sample and 2 biological samples from 1/2 to 1/32 in NaCl 0.9% solution and in urine pool of 5 healthy subjects, respectively. Control sample was Nephrocheck liquid control (high level) with concentration equal to 30.5 $(ng/mL)^2/1000$. Biological samples belonged to two ICU patients with AKI associated to cardiac surgery, had a Nephrocheck AKI score as follows: patient A= 1.87 $(ng/mL)^2/1000$, patient B= 0.41 $(ng/mL)^2/1000$. Then, we compared each point of dilution to its mathematical derivative (see figures 5-8), estimating the propagation of the systematic error simulated. Nephrocheck AKI score reference range associated to No AKI risk (0.002-0.3) (ng/

mL)^2/1000 and from a healthy and pathological population was analyzed; hence, analytical features of Nephrocheck test kit was critically taken into consideration.

2.1 Model of differential equation and Nephrocheck variation rate

It is well known that the differential equation model can be applied for the study of the biological system [10]. Differential equation is a relationship between a function and one of its derivatives. We calculated the first derivative of the function to investigate the rate of change of Nephrocheck AKI score calculation formula: (ng/mL) 2/1000. This is the measure unit of each Nephrocheck test as product of concentration of the two biomarkers: [TIMP-2]⊠ [IGFBP-7].

Hence, we investigated the influence of measure unit on a systematic error propagation. We observed clearly that (ng/mL)^2/1000 is a power function then if Y= X^2 = (ng/mL)^2/1000, then the differential equation will be: $dY/dX= dX^2/dX= D\ (ng/mL)^2/1000= DX^2 = 2X^{2-1}= 2X$. This means that the propagation of any error will follow a linear relationship (dY/dX= 2X), that is, the measure unit will contribute to the error propagation then to the analytical variability.

3. Results

For clinical characteristics and type of cardiac surgery of patients from both NGAL and Nephrocheck studies, we referred to the previous works on plasma consecutive NGAL measurements [7] and Nephrocheck after cardiac surgery [8] respectively. Here, we described results of urinary biomarkers to take into account pre and post-analytical pitfalls that can occur in detecting early AKI after cardiac surgery.

3.1 Urinary NGAL diagnostic accuracy and pre-analytical variability

Urinary NGAL results (pre-surgery) that are referred to the sampling time before operation (pre-uNGAL) as well as for plasma NGAL [7] results, did not show any association to AKI (p= 0.79). Instead, post-uNGALs showed a significant association to AKI with a cut-off= 29 ng/mL even if with modest sensitivity= 0.64 and specificity = 0.67 (p=0.016). Receiver operating characteristic curve revealed an AUC= 0.66 (Fig 1). We tried to correct post-NGAL in urinary samples through the corresponded urinary creatinine (uCrea) but we did not achieve significant results (p=0.25). Morever, we studied the uNGAL increases to detect potential acute damage according to an expert opinion [9], considering two consecutive measurements (pre -post uNGALs). Considering a biomarker increase of at least 50 ng/mL, we did not reach any sensitivity improvement for AKI detection. Instead, a significant association between the uNGAL increases and AKI severity was achieved (p=0.02).

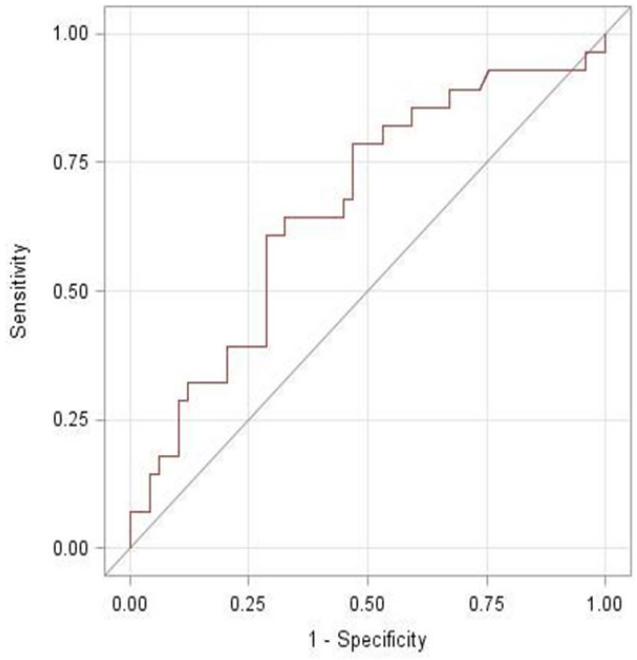

Figure 1 | ROC curve of urinary NGAL test (post-surgery)

In summary, for the urine NGAL and creatinine determinations (N=32) we obtained the follow results (median and interquartile range): pre-uNGAL= 18.3 ng/ml (7.7-23.7), pre-uCrea= 95.5 mg/dL (51.75-142.25); post-uNGAL= 19.2 ng/mL (3.8-86), post-uCrea= 12.4 mg/dL (7.8 -26). The first outcome illustrated that there was not any increase of uNGAL (pre-post surgery) with low uCrea concentration post-surgery, considering critically ill patients in intensive care unit (ICU). Urinary determinations were retrospectively analyzed to investigate possible causes of test inaccuracy. We plotted uNGAL versus uCrea concentrations (Fig 2) showing that 93% of results have fallen under 50 mg/dl of urine creatinine. Many uCreas had very low concentrations under 50 mg/dL, considered as graphical limit, close to the lower reference limit of a healthy population equal to 40 mg/dL. This finding suggests a possible dilution of urinary samples with a probable

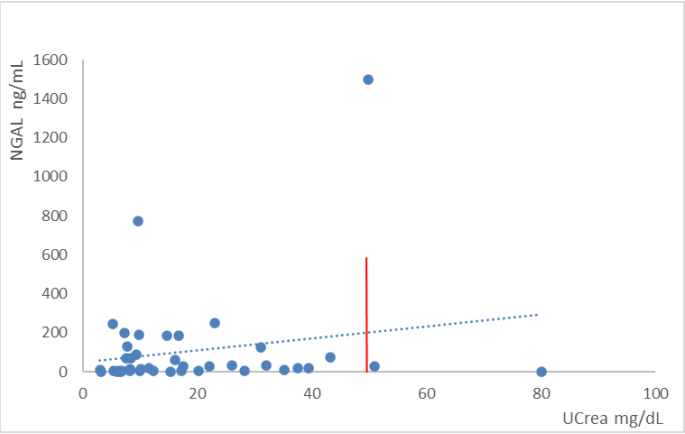

Figure 2 | Analysis of uNGAL and uCrea concentrations. Red line indicates uCrea concentration= 50mg/dL. uCrea as dilution marker showed that many biomarker concentrations falled under this line.

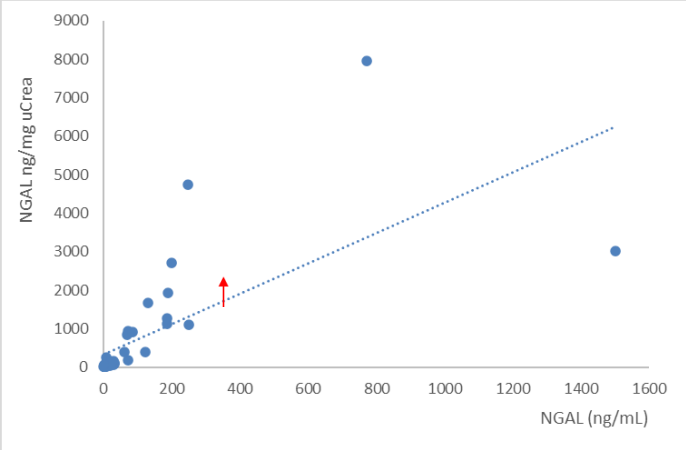

Figure 3 | uNGAL and uNGAL/uCrea correction results. Red arrow indicates the uNGALs trend to fall over the interpolation line with very high uNGAl values.

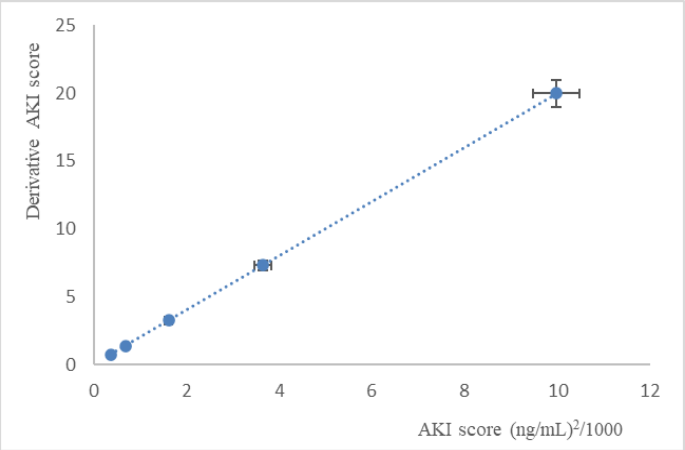

Figure 5 | Comparison between [TIMP-2]-[IGFBP-7] level and its derivative from control sample. (Error bars expressed as percentage)

interference or high pre-analytical variability. In fact, we tried to analyze all urine data by uCrea correction (Fig 3). Results showed a non-linear relation and mostly dispersion of data as uNGAL concentration increases. However, three patients reached a clinical significance for AKI diagnosis considering uNGAL increases >50 ng/mL. Otherwise, many uNGALs corrected by uCrea showed uNGAL values (N=10) not-specifically raised for AKI (Fig 3). These results showed that the NGAL correction by uCrea generates uNGAL ng/mg uCrea values too high (see Y-axis, Fig 3) for a useful clinical interpretation.

3.2 Nephrocheck AKI score results and pre/post-analytical variability

3.2.1 Pre-analytical variability

Results published in the previous study, illustrated the follow ROC curve parameters: AUC= 0.64, (confidence interval= 0.5- 0.77) (p= 0.048). Although, it was reach a statistical significance, diagnostic accuracy did not reach clinical significance to justify Nephrocheck test for AKI prevention, considering our study design and patient population [8].

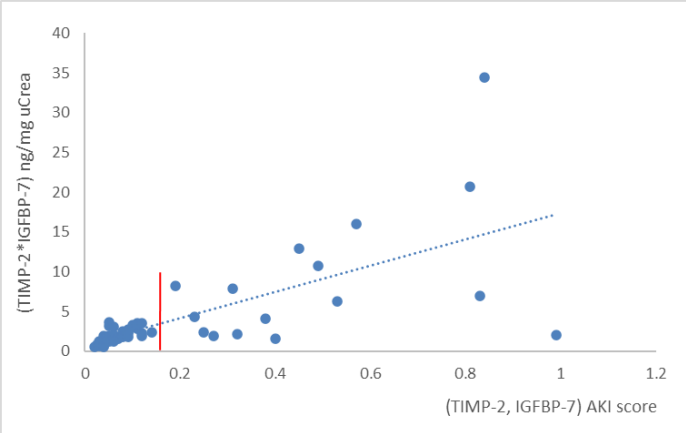

Figure 4 | Interpolation of [TIMP-2]-[IGFBP-7] AKI score with the same values corrected by uCrea.
Better correlation was achieved for AKI score under 0.18 (red line).

Morever, analysis of [TIMP-2]-[IGFBP-7] AKI scores corrected by uCrea revealed the following results:

Y= 16.589X+0.8134, R^2= 0.46, R= 0.67 (Fig. 4). The present equation suggested an overestimation of biomarker values corrected, (see high [TIMP-2]-[IGFBP-7]ng/mg uCrea values) with a possible systematic error. Similarly to the uNGAL results, Nephrocheck AKI score seemed to be affected by pre-analytical variability as well as sample dilution (results published by l'Acqua et al. [8]). Correction of [TIMP-2]-[IGFBP-7] concentration through uCrea did not add significant improvements for AKI detection, instead generated uninterpretable results. We observed that only for low Nephrocheck AKI scores referred to physiological conditions (Nephrochek AKI score < 0.2) could be obtained a good correlation (Fig 4).

3.2.2 Post-analytical variability

Model of differential equation allowed us to know better post-analytical variability and the behavior of result expression to prevent any error propagation. We supposed that the measure unit $(ng/mL)^2/1000$ of [TIMP-2]-[IGFBP-7] concentrations affects clinical data then introduces a post-analytical variability due to the amplification of analytical and pre-analytical errors. Experimental (Nephrochek AKI score) and mathematical (Derivative Nephrocheck AKI score) data obtained from liquid control and biological samples were compared. As expected, for the three different evaluations, mathematical derivative of biomarker measurements was significantly associated to the biomarker concentration. In fact, D $(ng/mL)^2/1000$= 2X, then the variation rate follows a linear function (see Fig 5, Fig 6, Fig 7) with coefficient of correlation R=1. Thereby, the error propagation, as mean variation rate, increases as [TIMP-2]-[IGFBP-7] measure increases, linearly. This finding underlined that the measure unit of Nephrocheck test: $(ng/mL)^2/1000$ is a power function. In addition, we plotted the

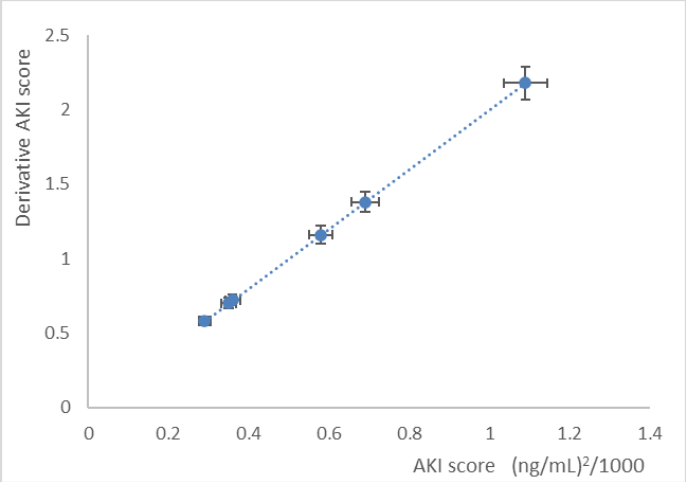

Figure 6 | Comparison between [TIMP-2]-[IGFBP-7] level and its derivative from Patient A. (Error bars expressed as percentage)

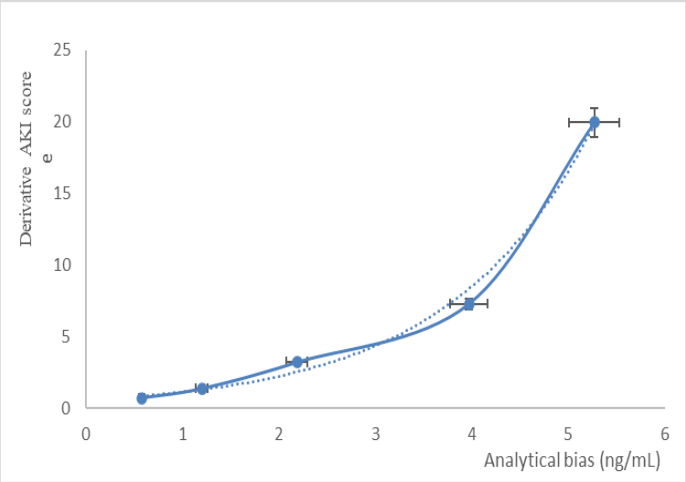

Figure 8 | Graphic from control sample illustrates systematic error (absolute bias values) and relative derivatives. Note that for analytical bias higher than almost 4 ng/mL, trend becomes exponential. (Error bars expressed as percentage)

systematic error calculated from the experiment of pathological control sample considering the analytical bias (Fig 8.) Results showed: Y= 0.5954 (e) exp 0.665X with R^2= 0.98 and R= 0.99. Data demonstrated an exponential relation between the Derivative AKI score and the analytical bias calculated as difference (expected value – observed value). Analysis of curve suggests, however, that the exponential trend occurs only for high bias values (Fig 8.)

3.2.2.1 Comparison of Nephrocheck score versus NGAL for the mathematical derivative

NGAL biomarker with measure unit equal to (ng/mL) follows a linear function like most of clinical measurements in laboratory medicine based on a single concentration. In fact, if we applied the differential equation to the NGAL with measure unit (ng/ml) we obtain as follows: Y= ng/mL=X, then the differential equation dY/dX=D (ng/mL)= X^{1-1} =X^0 =1, which is a constant. In summary, dY/dX of Nephrocheck is equal to 2X and dY/dX of NGAL is 1. This means that uNGAL testing, is not affected by the error propagation or variation change due to the result calculation or measurement expression.

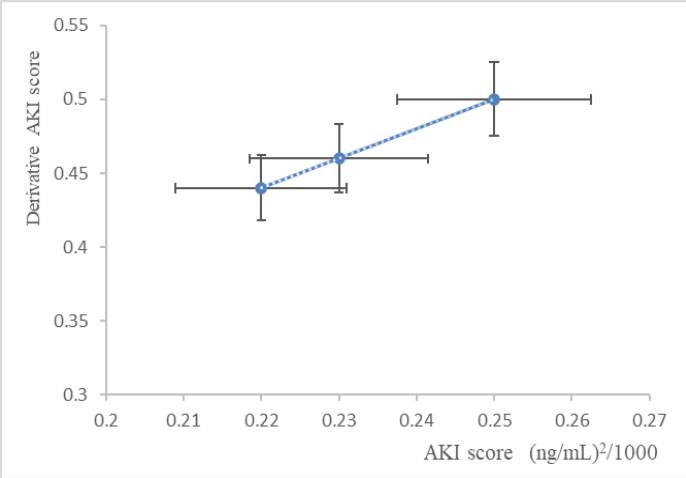

Figure 7 | Comparison between [TIMP-2]-[IGFBP-7] level and its derivative from Patient B. (Error bars expressed as percentage). (See the high dispersion of measures due to very low biomarker levels)

3.2.2.2 Analytical features of Nephrocheck AKI score and possible improvements

About the performance characteristics of Nephrocheck test, we observed that normalization of biomarker concentrations $(ng/mL)^2$ divided for 1000 provides a numerical reduction of biomarker levels as fraction of 1. For this reason, Nephrocheck AKI score cut-off equal to 0.3, extensively used in clinical trials, may be easily misinterpreted among different groups of patients. In fact, we considered the measure unit or fraction: $(ng/mL)^2/1000$ as an attempt to show both [TIMP-2]-[IGFBP-7] concentrations and not a true and necessary analytical normalization. The effect of this transformation is a detection and quantification limit of Nephrocheck score equal to 0.002. This means a quantification limit unsuitable for clinical use as a very low fraction of unit 1. Our observations noted that, to reduce the analytical variability should be used a concentration product of the two biomarkers [TIMP-2]-[IGFBP-7] in linear form as (ng/mL). Then, we could assume a transformation through a square root of the concentration product [TIMP-2]-[IGFBP-7] $(ng/mL)^2$, as follows:

$[(ng/mL)^2]^{1/2}$ =ng/mL. In instance, if we have a [TIMP-2]-[IGFBP-7] concentration product equal to 300 $(ng/mL)^2$, we can obtain a cumulative concentration equal to 17.32 ng/mL through its square root, corresponded to Nephrocheck score equal to 0,3 $(ng/mL)^2/1000$. This could allow a numerical result suitable for laboratory practice and not affected by the error propagation. However, we highlighted as a declared post-analytical variability of Nephrocheck AKI score the reference range expressed as $(ng/mL)^2/1000$: (0,04–2.25) and

(0,05–2.20) for apparently healthy subjects and for subjects with stable chronic morbidities respectively.(data published from the Nephrocheck test kit package insert). If we consider the reference range (0,04–2.25) expressed as (ng/mL)^2/1000, we can obtain, through a square root and removing the division of a 1000, the expression (ng/mL) equal to (6.32-47.73). This reference range should determine a reduction of measure dispersion, supporting the use of a linear unit (ng/mL).

4. Discussion

AKI diagnosis is currently defined by the serum creatinine and urine output results, according to KDIGO guidelines. Nevertheless, both serum creatinine and urine output are tardive for an appropriate early AKI diagnosis and to start therapy. In the last years, in this scenario, much attention has focused on novel renal biomarkers as tools to detect kidney injury, or as recently described, on acute tubular damage or biochemical stress detection. At the same time, the extra-analytical phase in laboratory medicine (pre and post-analytical variability) (TTP) became really an issue to be also solve for new biomarkers, with researches and scientific interest worldwide. Our study has pointed out the pre and post-analytical phases of new renal biomarkers: urinary NGAL and [TIMP-2]-[IGFBP-7] proteins called Nephrocheck test. Indeed, we evaluated urinary biomarker results of two previous studies [7,8] conducted in our Center in predicting CSA-AKI, on an adult ICU population with high risk of renal impairment. Recently, it seemed that [TIMP-2]-[IGFBP-7] biomarkers provide better diagnostic outcomes to detect kidney stress than NGAL or other new promising tests [11,12]; nevertheless biomarker levels become significant 12-24 hours after clinical insult only. In addition, studies with different patient settings gave controversial information for biomarker application in medical practice; in summary requiring more evidences [13]. Our results on urinary biomarkers were obtained with an experimental protocol based on a sampling time within 4 hours from arrival of the patients in ICU (post-biomarker), then 6 hours after clinical insult, approximately. Reason of an early biomarker sampling was to achieve timely information to predict CSA-AKI. In fact, we reached good outcomes in a first study conducted on plasma samples, considering two consecutive NGAL measurements [7]. Instead, considering urinary samples of a subset of patients, both NGAL and Nephrocheck test were poor predictors of AKI (modest AUC) then not useful for clinical application. Analyzing urinary creatinine, as dilution marker, we showed that 93% of patients had urine creatinine concentration under 50 mg/dL, according to previous study [8] in the same clinical setting and study design. Indeed, in accordance with other works, we suggested the possibility of a sample dilution, with the advice that the results should be corrected for the fluid variations [8,14,15]. As suggested by RG Hahn [14], Nephrocheck AKI score might increase when urine was concentrated and likewise decrease when urine spot was diluted, generating an overlap of results among patient RIFLE groups. Accordingly, in the present investigation we sought to correct urinary biomarkers by the uCrea, because of a urine spot could contain the effects of hemodilution or concentration, in instance during the diuretic therapy. Although these considerations, our results showed that both uNGAL and Nephrocheck AKI score had not any significant correction and useful result by uCrea. Furthermore, the graphics (Fig. 3-4) illustrated a data dispersion as biomarker concentration increases. In fact, Waikar S Sushrut et al. [16,17] demonstrated that biomarker normalization by uCrea may introduce a bias with an underestimation or overestimation of biomarker excretion rate depending on clinical context. In addition to pre-analytical variability, we evaluated post-analytical phase using a mathematical model and observations on the analytical features of Nephrocheck test. Firstly, differential equations revealed an error propagation due to the measure unit of Nephrocheck AKI score. Notably, Nephrocheck AKI score is an arithmetic product of two biomarkers: [TIMP-2]-[IGFBP-7] with generation of concentration square (ng/mL)^2. Then, every small error (pre-analytical or analytical) could be amplified by result expression (ng/mL)^2/1000, according to its mathematical derivative. We supposed that this finding might contribute to the inaccuracy of Nephrocheck test. Secondly, it is possible that the transformation of TIMP-2, IGFBP-7 concentrations to Nephrocheck AKI score, could introduce a high analytical variability around the AKI risk cut-off=0.3, causing an overlap of different patient groups with suspect of AKI. Analyzing the measure unit (ng/mL) 2/1000, we suggested that an expression of the TIMP-2, IGFBP-7 concentrations as cumulative concentration in linear form (ng/mL) may reduce the post-analytical variability. Remains to plan further studies to verify whether [TIMP-2]-[IGFBP-7] cumulative concentration may improve diagnostic outcomes for the AKI risk assessment. In conclusion, we thought that a standardized sample collection and a better expression of Nephrocheck test could represent a worthy step to AKI prediction in ICU critical patients.

5. Concluding Remarks

The urgent need to have new renal biomarkers inspires the effort of researchers in proteomic basic studies as well as in translational and clinical trials. Recent works by expert nephrologists suggest the AKI biomarker application in clinical practice through algorithms or consecutives measurements (AKI biomarker curves) [11,18]. In the last months, notably, another cause of AKI was described: Covid -19 associated AKI, requiring important insights to prevent organ failure [19]. Furthermore, a reproducible diagnostic accuracy and validation of new biomarkers is crucial in own laboratory and clinical practice. In the present study we evaluated urinary NGAL and [TIMP-2]-[IGFBP-7] with an early timing (4-6 hours after renal damage) and

consequently the outcomes have not been good as expected. According to other authors, we indicated that, in critically ill patients underwent cardiac surgery, pre-analytical conditions were characterized by concentration/dilution of urinary samples. Advances in timed urine collections [16] and standardization of pre-analytical procedures may add new findings. Probably, our sampling time too early, determined sample dilution, because of high hemodynamic changes in critical post-surgical patients. Another issue was the analytical expression of Nephrocheck test or measure unit. We demonstrated that error propagation and Nephrochek test uncertainty may be reduced using a linear measure unit as ng/mL. Finally, new urinary biomarkers might be improved for clinical use whether pre and post-analytical variabilities will achieve a meaningful reduction, allowing a medical help for AKI prevention.

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