A DIAGNOSTIC ASSAY FOR IL-33 AND SST2 AS BIOMARKERS FOR ACUTE KIDNEY DISEASE

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By

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On my honor as a University student, I have neither given nor received unauthorized aid on this assignment as defined by the Honor Guidelines for Thesis-Related Assignments.

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A Diagnostic Assay for IL-33 and sST2 as Biomarkers for Acute Kidney Disease

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Abstract

One of the most common complications during cardiac surgery is acute kidney injury (AKI), occurring in as much as 31% of patients. In this condition, the kidney undergoes an abrupt reduction in function. If the diagnosis and treatment of AKI are prolonged, kidney cells will experience greater damage and the chance for recovery decreases. Currently, AKI is diagnosed with a blood test to measure creatinine levels or by measuring urine output over several hours. However, creatinine tests are imperfect due to the lack of specificity and sensitivity and cannot indicate AKI in the early stages. Biomarkers and rapid urine assays are needed to detect AKI earlier and more accurately. This project focuses on two biomarkers for AKI: Interleukin 33 (IL-33) and soluble Suppressor of Tumorigenicity 2 (sST2). Both IL-33 and sST2 are involved in immune cell recruitment and have been found to be elevated in patients with poor cardiac surgery outcomes and kidney damage. This project aimed to develop an assay to effectively detect IL-33 and sST2 in 24-hour cumulative urine samples from cardiac surgery patients and correlate them with AKI diagnosis. First, tests were run on cardiac surgery patient samples and were inconclusive in establishing the analytes as biomarkers to diagnose AKI. Then, assays were run to assess whether the analytes degraded urine samples upon 24 hours of storage and multiple freeze-thaw cycles. Although results showed the analytes likely did not degrade in urine samples, further work must be done to measure the analytes in urine samples at earlier time points or in plasma.

Keywords: Acute kidney injury, ELISA, Diagnostic test, Cardiac surgery

Introduction

Acute Kidney Injury after Cardiac Surgery

As much as 31% of patients undergoing cardiac surgery are diagnosed with acute kidney injury (AKI)[1]. AKI occurs when the kidneys experience an abrupt reduction in renal function. During cardiac surgery, ischemia is often the cause of this disease as the amount of blood and oxygen delivered to the kidney is reduced. This is due to cardiac surgery patients' blood flow being rerouted through a cardiopulmonary bypass, colloquially known as the heart-and-lung machine, during the surgery[2]. As the diagnosis and treatment of AKI are delayed, renal cells can experience extensive damage and death, reducing the possibility for the kidneys to recover. In clinical settings today, AKI is diagnosed by administering blood and urine tests to measure creatinine levels or a urine output test to measure the amount of urine produced by the body over the course of 24 hours[3]. As creatinine is a natural product of metabolism, its measurements produce imperfect results due to the lack of specificity and sensitivity. Work by Swedko et al. in 2003 found

that creatinine only had a 12.6% sensitivity for detecting renal failure[4]. Therefore, the early stages of AKI cannot be diagnosed through creatinine tests. In order to accurately identify AKI earlier in patients, the use of biomarkers and rapid urine assays will be vital in clinical settings.

IL-33 and sST2 axis

This project focused on Interleukin 33 (IL-33) and soluble Suppressor of Tumorigenicity 2 (sST2), which previous research has identified to have the potential to be AKI biomarkers. IL-33 is highly expressed by vascular endothelial cells and is released in the regions of damaged kidney tissue to recruit immune cells[5]. Among the recruited immune cells are regulatory T cells that mediate the inflammatory response in the damaged kidney tissue[6]. Previous research into this cytokine also indicates that IL-33 responds to ischemia-reperfusion injury, which cardiac surgery patients can experience and cause AKI[6]. ST2 is the receptor of IL-33 and is expressed in two isoforms, ST2L, the membrane-bound form that is expressed on specific immune cells, and sST2, the shorter soluble form which acts as a “decoy” to sequester IL-33 and reduce fibrotic and apoptotic activity in damaged nephrotic tissue. Studies found elevated levels of sST2 in cardiac surgery patients with kidney damage and poor surgical outcomes[7,8].

IL-33 and sST2 as AKI Biomarkers

As a result of the previous research conducted, it is hypothesized the concentrations of IL-33 and sST2 can serve as a sensitive and accurate indicator for AKI. The goal of this project was to develop an assay that can effectively detect IL-33 and sST2 in urine samples of cardiac surgery patients. The analyte concentrations can then be compared to the AKI clinical diagnosis patients of the samples received. If this correlation between the urine assay results and the known patient diagnoses is proven, this information can then be utilized to validate IL-33 and sST2 as biomarkers. By evaluating IL-33 and sST2, the urine assay can also help to analyze renal damage and immune-cell infiltrates, which can provide insight into the most effective form of treatment in clinical settings.

Materials and Methods

Patient Samples

The study protocols were approved by the Institutional Review Board at the University of Virginia prior to sample collection. The urine samples utilized in this project were obtained from consenting adult patients who underwent two different types of cardiac surgery: coronary artery bypass graft surgery (CABG) and/or valve repair. Patients on immunosuppressive medication were excluded. All the samples were de-identified without any patient descriptors and the AKI status of the samples were kept blinded during the experiments to ensure the data analysis is objective. Within the sample pool, the AKI diagnosis and status varied ranging from no AKI diagnosis to stage 2 AKI. Historical data on the analyses of Insulin-Like Growth Factor Binding Protein 7 (IGFBP7) and Tissue inhibitor of metalloproteinases 2 (TIMP2), two components of FDA-approved biomarker Nephrocheck for AKI were also used as comparators.

Analyte Measurements

In order to measure and evaluate the concentrations of IL-33 and sST2 individually, an enzyme-linked immunosorbent assay (ELISA), particularly a sandwich ELISA, was the primary method experimental technique for this project. ELISA kits specific to IL-33 and sST2 were obtained from the cell biology research tool company, R&D Systems. Each kit included capture and detection antibodies, streptavidin-horseradish peroxidase (HRP), and human IL-33 or sST2. In this protocol, a capture antibody is first coated onto the plate overnight. After a blocking step with 1% bovine serum albumin (BSA) in phosphate-buffered saline (PBS) to prevent nonspecific binding, samples containing the analyte of interest are added. The capture antibody binds a specific epitope on the analyte. A biotinylated detection antibody is then added; this binds a different epitope on the same analyte. Streptavidin-HRP is then added and binds to the biotinylated detection antibody so that it can react with TMB substrate to produce a colored product that can be measured with an absorbance microplate reader. This project conducted three rounds of experimental testing for IL-33 and sST2. The first round of experiments tested the biomarker concentrations in cardiac patient samples. These samples were diluted at factors of 1:2, 1:4, and 1:8.

Analyses for the Stability of Analytes in Urine

The objective of the next experiment was to

assess the effect of time and temperature on the stability of the biomarkers within urine samples. This allowed for the assessment of whether any compounds in the urine caused analyte degradation, and whether specific storage conditions mitigate this. For each biomarker, urine samples that did not have AKI were pooled and aliquoted into eight samples. Four of these samples were stored at 4°C and the remaining samples were stored at room temperature, approximately 2°C. Each set of samples was spiked with the biomarker 24 hours, 12 hours, 3 hours, or 1 hour before the samples were tested. The second set of experiments evaluated the biomarker stability under various freeze-thaw cycles. Pooled urine samples without AKI were also utilized for these experiments. The samples were spiked with IL-33 or sST2 the first time they were taken out of the freezer and thawed. The samples were returned to the freezer until completely frozen and then taken out to thaw. This cycle would be repeated until the samples reached 1, 2, or 3 freeze-thaw cycles. After each experiment was conducted, a 96 Microplate Reader was utilized to measure the absorbance at two wavelengths, 450 nm and 570 nm. The delta absorbance at these wavelengths was calculated and utilized for further analysis. This data was then imported to GraphPad Prism 9, an analysis software program, to interpolate the data and generate the figures shown in this report.

Results

Testing of Cardiac Surgery Patient Samples

AKI Diagnosis	Patient Sample Number
No AKI	B4, B5, B7, B8, B10, B11, B12, B13, B14, B15, B16, B19, B20, B21, B22, B23, B24, B25, B26, B27, B29, B30, B31, B32, B34, B35, B36, B37, B38, B39, B40, B41, B42, B43, B45
Stage 1 AKI	B2, B3, B9, B17, B18, B28, B33, B41, B44
Stage 2 AKI	B1

Table 1. AKI diagnosis status of 44 cardiac surgery patient samples.

A total of 44 samples from cardiac surgery patients were tested for both IL-33 and sST2. The samples were diluted at 1:2, 1:4, and 1:8. The concentration of the samples was calculated from the absorbance and then compared to the diagnosis patients of the sample received after cardiac surgery. As shown in Table 1, 9 patients were diagnosed with stage 1 AKI, and 1 was diagnosed with stage 2 AKI. Assays on the analytes were done while blinded to the patients' AKI status.

Reliable measurements for IL-33 were only able to be made for patients B1-B5 and B7-15. As seen above in Figure 2, IL-33 concentrations for this set of patient samples were highly variable across different dilution factors and did not strongly differ between patients with or without AKI. In particular, IL-33 concentration in the 1:4 diluted urine varied from 38.83 pg/mL to 73.53 pg/mL for AKI patients, while it varied from 26.19 pg/mL to 141.17 pg/mL for non-AKI patients. Patient B1, who was diagnosed with stage 2 AKI, actually had the lowest IL-33 concentration out of all the AKI patients at 38.83 pg/mL. Additionally, the second and third sets of patient samples (patients B16-B30) produced absorbances so low that extrapolation from the linear standard curve calculated negative concentration values. This is likely because the IL-33 concentrations in these diluted samples were below the minimum detection limit of the R&D Systems ELISA kits that were used for the assay.

The sST2 concentration of the stage 1 AKI samples ranged from 9.13 pg/mL to 750.03 pg/mL (Figure 2). There was one sample with a patient diagnosis of stage 2 AKI, with an average sST2 concentration of 631.65 pg/mL at a 1:4 dilution (Figure 2). This concentration was less than the concentrations of the stage 1 samples, which was unexpected as it was predicted that the sST2 concentration would increase as the severity of AKI increased, similar to creatinine increased, similar to

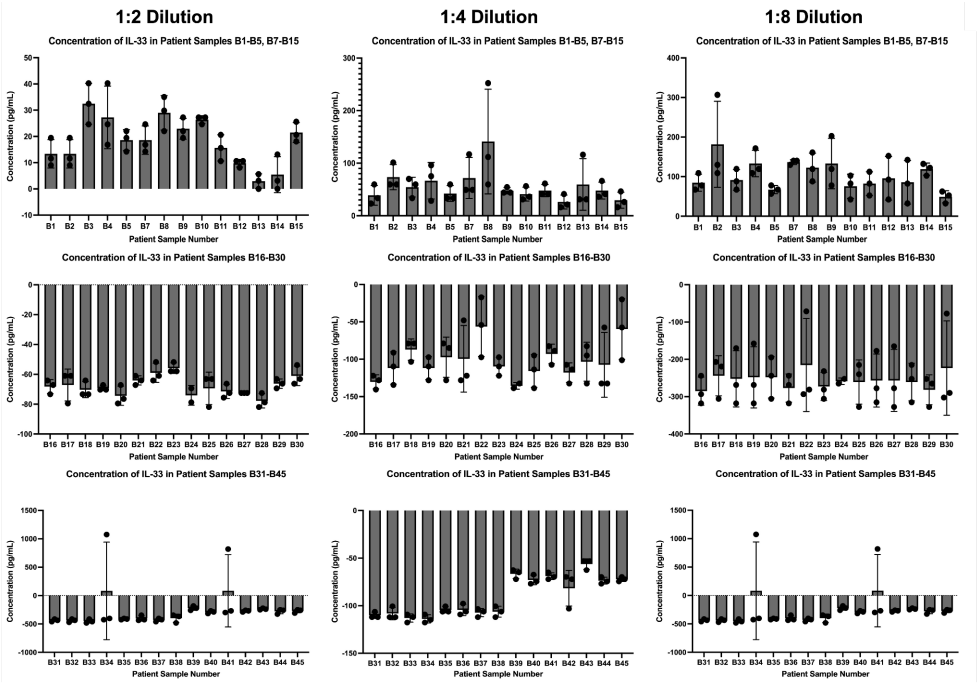

Figure 1. The concentration of IL-33 found in cardiac surgery patient samples. A total of 44 samples were diluted into 1:2, 1:4, and 1:8 dilutions and tested in triplicates. The concentration values were extrapolated using a best-fit linear curve. The points represent individual values and bars represent the mean of triplicates with standard deviation error bars. Absorbances for the 2nd and 3rd patient sets (rows 2 and 3) were likely below the minimum detection limit, resulting in the extrapolation from the standard curve producing negative concentration values.

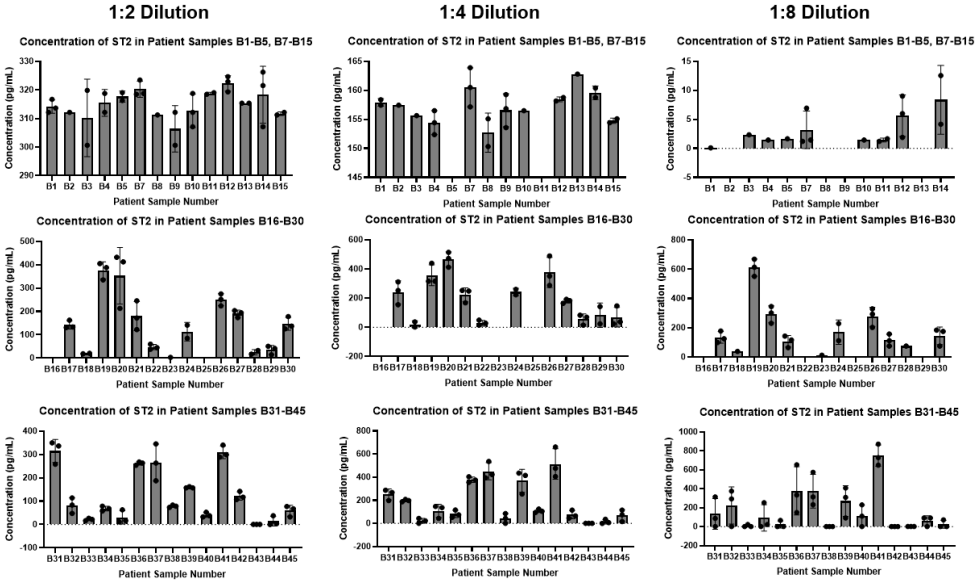

Figure 2. The concentration of ST2 present in cardiac surgery patient samples. A total of 44 samples were diluted into 1:2, 1:4, and 1:8 dilution and tested in triplicates. The concentration values were interpolated using the best fit of a sigmoidal curve. The points represent the individual values and the bars represent the mean of the triplicates with standard deviation error bars.

creatinine.

Along with the AKI status of each patient, the concentrations of two additional biomarkers, Tissue Inhibitor of Metalloproteinase-2 (TIMP2) and Insulin-like Growth Factor Binding Protein-7 (IGFBP7), were recorded. These biomarkers are currently utilized in NephroCheck, which is a clinical test for early stages of AKI approved by the US Food & Drug Administration in 2014[9]. The AKI patient concentrations for TIMP2 (1:4 dilution) and IGFBP7 (1:100 dilution) were compared to the sST2 concentration to determine a correlation between these biomarkers. A correlation test was conducted between IGFBP7 and sST2 for all dilutions. The test

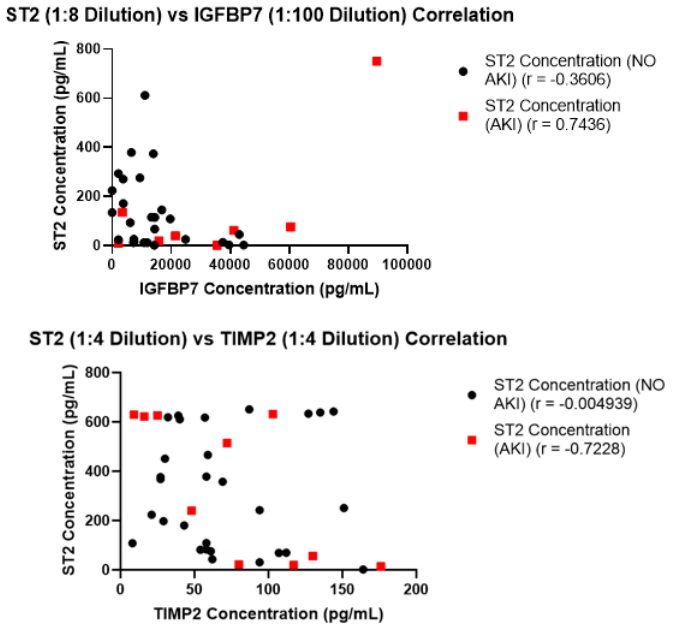

Figure 3. Concentration correlation between ST2 and IGFBP7 (top graph) and ST2 and TIMP2 (bottom graph). These comparisons produced the strongest and statistically significant correlation coefficient (r).

found correlation coefficient values of -0.2088, -0.06587, and 0.7436 for 1:2, 1:4, and 1:8 dilutions, respectively (Table 2). A two-tailed statistical test was conducted and determined the positive correlation between IGFBP7 and the 1:8 dilution of sST2 was statistically significant with a p-value of 0.0344 (Figure 3). The correlation test between TIMP2 and sST2 also yielded negative correlation coefficients of -0.7028, -0.7228, and -0.1615 for 1:2, 1:4, and 1:8 sST2 dilution, respectively. Statistical significance was found in the 1:2 and 1:4 sST2 dilution with p values of 0.0234 and 0.0182, respectively (Figure 3).

Stability of Analyte Over Time and Temperature

Urine samples spiked with IL-33 and sST2 were subjected to various storage times and temperatures as indicated above. For each sample, serial dilutions were plotted and a 4-parametric logistic equation was fitted. Curves of serial dilutions of each condition plotted alongside the standard curve are seen below in Figure 4. There was no consistent visual difference between the curves of different IL-33 storage conditions and the standard curve. This suggests that variation in storage conditions of IL-33-spiked urine has little to no effect on the detectability of IL-33 with the available ELISA kits. As a result, this also suggests that compounds

Correlation Test	Correlation Coefficient	R^2	P-value
IGFBP7 (1:100 Dilution) vs. ST2 (1:2 Dilution)	-0.2088	0.04360	0.5626
IGFBP7 (1:100 Dilution) vs. ST2 (1:4 Dilution)	-0.06587	0.004339	0.8565
IGFBP7 (1:100 Dilution) vs. ST2 (1:8 Dilution)	0.7436	0.5530	0.0344
TIMP2 (1:4 Dilution) vs. ST2 (1:2 Dilution)	-0.7028	0.4939	0.0234
TIMP2 (1:4 Dilution) vs. ST2 (1:4 Dilution)	-0.7228	0.5225	0.0182
TIMP2 (1:4 Dilution) vs. ST2 (1:8 Dilution)	-0.1615	0.02607	0.7025

Table 2. This table provides the correlation coefficient and R^2 of the correlation tests between the AKI patient concentrations of IGFBP7 and ST2 or TIMP2 and ST2. A two-tailed statistical test was conducted for each correlation group to obtain the p-value.

present in the urine do not have a degrading or denaturing effect on IL-33.

The stability of sST2 was also evaluated under various incubation times and storage conditions. Although there was variation in the curves among incubation times, there was no statistically significant difference (Figure 4). The change in storage temperature had the least variation in the detection of sST2. The curves indicated the greatest difference between room temperature and 4°C conditions was during the 24-hour incubation time with an average difference of approximately 0.0438 (Figure 4).

The results of this experiment suggest time and temperature do not have a statistically significant effect on the detection of either IL-33 or sST2 during the ELISA testing.

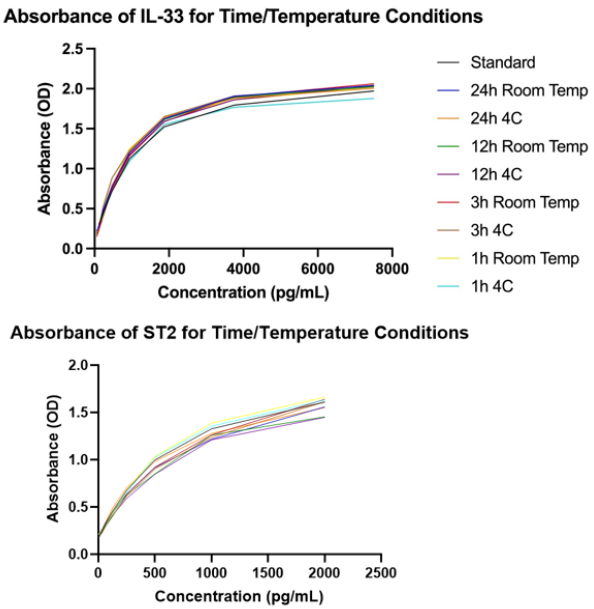

Figure 4. The average absorbance of urine samples spiked with IL-33 and ST2 under different incubation and storage conditions. The samples were spiked at 1, 3, 12, and 24 hours before the ELISA test. The samples were also stored at room temperature (20°C) or in a cold room (4°C).

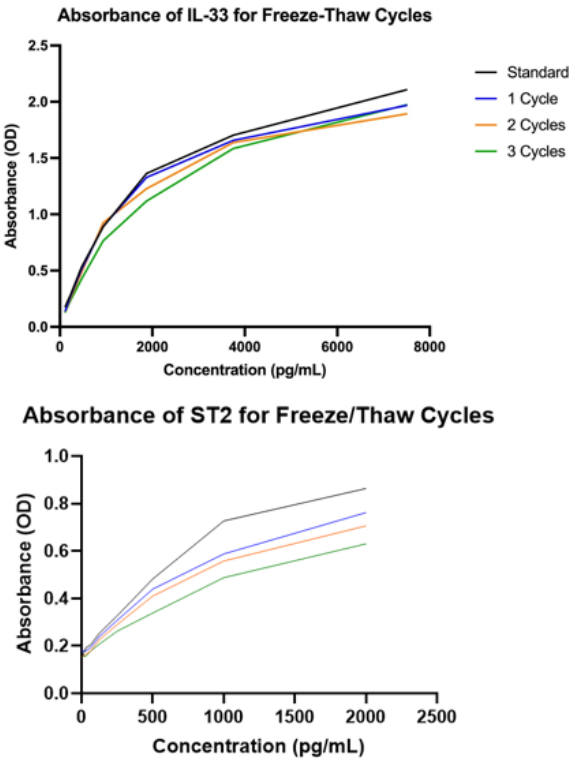

Figure 5. The average absorbance of urine samples spiked with human IL-33 and ST2 that have undergone 1, 2, and 3 freeze thaw cycles. Each sample is a 1:4 dilution of urine to reagent diluent (1% bovine serum albumin (BSA) in phosphate buffered saline (PBS)) and underwent a serial dilution. The samples taken out of the freezer to thaw and spiked with ST2. Then the samples were returned to the freezer to complete the necessary freeze thaw cycles.

Stability of Analyte Over Freeze-Thaw Cycles

Urine samples spiked with IL-33 and sST2 were also subjected to 1, 2, and 3 freeze-thaw cycles as indicated above. The absorbance was plotted against the concentration in Figure 5. It can be visually seen that absorbance slightly drops as spiked samples are subjected to freeze-thaw cycles, however there is no significant reduction in IL-33 detection with repeated cycles. As such, data shows a general trend suggesting that subjecting urine to freeze-thaw cycles may slightly worsen IL-33 detectability, however not by a significant amount.

The data obtained from this experiment indicate the number of freeze-thaw cycles is statistically significant to the accuracy of the detection of sST2 (Figure 5). When compared to the standard, which did not undergo any freeze-thaw cycles, the absorbance decreased as the number of freeze-thaw cycles increased (Figure 5). Therefore, the detection of sST2 decreases with each cycle, reducing the accuracy of the results. Additionally, the absorbance of the standard and samples with 1 and 2 freeze-thaw cycles were statistically greater than the samples that underwent 3 cycles (Figure 5). This demonstrated that 3 freeze-thaw cycles caused the sST2 concentrations to be significantly inaccurate. Based on the results of this experiment, it was concluded that the urine samples should not exceed two freeze-thaw cycles as the detection of sST2 will significantly decrease the accuracy of the ELISA.

Discussion

In the first phase of this project, patient urine samples were tested for concentrations of IL-33 and sST2. As seen above, the results were inconclusive in connecting analyte concentrations with AKI diagnosis across all dilution factors and both analytes. Statistically significant correlations were found between sST2 and known AKI biomarkers TIMP2 and IGFBP7. It was important for these biomarkers to be tested against sST2 because they are clinically approved in NephroCheck. It must also be noted that the IGFBP7 and TIMP2 values were also not in complete agreement with the clinical diagnoses of AKI in this subset of patients. Upon analysis, a statistically significant positive correlation was found between sST2 and IGFBP7 and a negative correlation between sST2 and TIMP2. However, these results are not extensive enough to confirm the relationship is strong enough for clinical testing. As such, the question arises: did the storage conditions of urine samples have any effect on the detectability of both IL-33 and sST2 in the samples?

Firstly, serial dilutions of pooled urine spiked with IL-33 and sST2 were plotted against the standard curve after being subjected to storage for up to 24 hours at either 4°C or 20°C (room temperature). As seen above, there was no significant difference across different storage conditions for either analyte. Prior work has also shown that the detectability of similar cytokines is not significantly impacted by different storage times and temperatures. Kenis et al. found that cytokines such as IL-6 and CC16 did not significantly reduce in concentration after several days of storage at either 4°C, 20°C, or 30°C[10]. Overall, these results prove that compounds present in the urine such as urea or uric acid do not degrade the analytes, regardless of storage conditions.

Secondly, the same analysis was performed for spiked urine samples subjected to multiple freeze-thaw cycles. As seen above, there is again

little effect on IL-33 detectability, but there was a significant effect on sST2. Freeze-thaw cycles were tested for their effect on the analytes as prior research shows that this can cause protein denaturation and affect detection by antibody-based assays. Prior work on myofibrillar protein (MFP) has found that freeze-thaw cycles can cause disruption of protein secondary and tertiary structure as well as protein aggregation that can affect detectability with a sandwich ELISA[11]. This is potentially the effect that worsens sST2 detection after several freeze-thaw cycles. However, this does not explain why IL-33 detection was not strongly affected by multiple freeze-thaw cycles. The previously mentioned work by Kenis et al. also observed that detection of similar cytokines was not impacted by freeze-thaw cycles.

In all, general trends from our work indicate that storage of the urine samples was unlikely to have had a strong effect on the detection of analytes in the patient samples. As such, the inconclusive results from the patient samples are likely tied to the limitations of this work. Firstly, testing was conducted on a low sample size (n=44), of whom only 10 had AKI. As a result, it was difficult to visualize any strong demarcation between patients who had AKI and those who did not. This small sample size of 10 AKI patients particularly made it difficult to establish meaningful conclusions from the correlation analysis between sST2 and TIMP2. Additionally, the only available patient samples were 24-hour pooled urine samples. However, such samples cannot provide insight into levels of biomarkers over the course of the 24 hours immediately following surgery. Based on urine studies, one potential scenario is shown below in Figure 6, where IL-33 and sST2 elevation may occur in the urine in the earlier hours after surgery, but are then diluted in the 24-hour pooled samples once levels drop. The analytes may instead be elevated in the plasma several hours after surgery. Secondly, since renal function is compromised in AKI patients, it is possible that proteins that are larger are unequally filtered and reabsorbed from the glomerular filtrate. Future work testing patient plasma samples may provide a more conclusive connection between the analytes and AKI diagnosis.

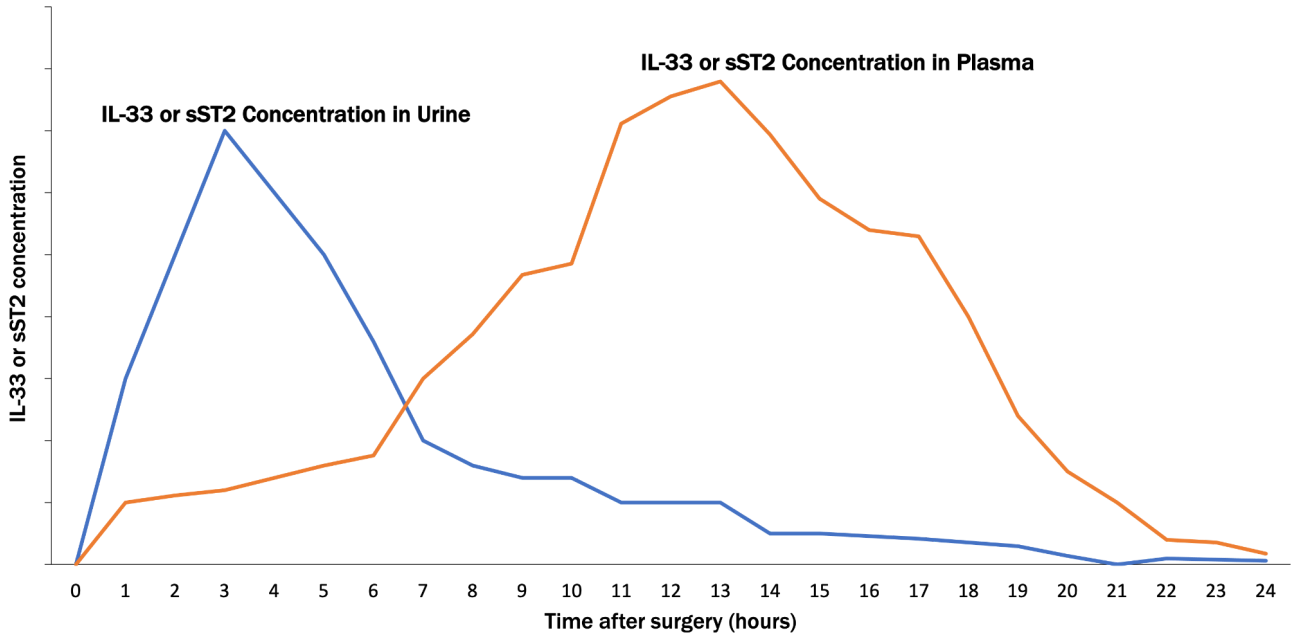

Figure 6. Graph showing a potential scenario for IL-33 and sST2 concentrations in urine and plasma after surgery. Such a scenario could explain why IL-33 and sST2 did not consistently correlate with AKI diagnosis in the 24-hour pooled samples that were tested.

Author Contributions and Notes

A.B. and A.S. equally contributed to the design, research, data analysis, and composition of this paper.

The authors declare no conflict of interest.

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Supplementary Information

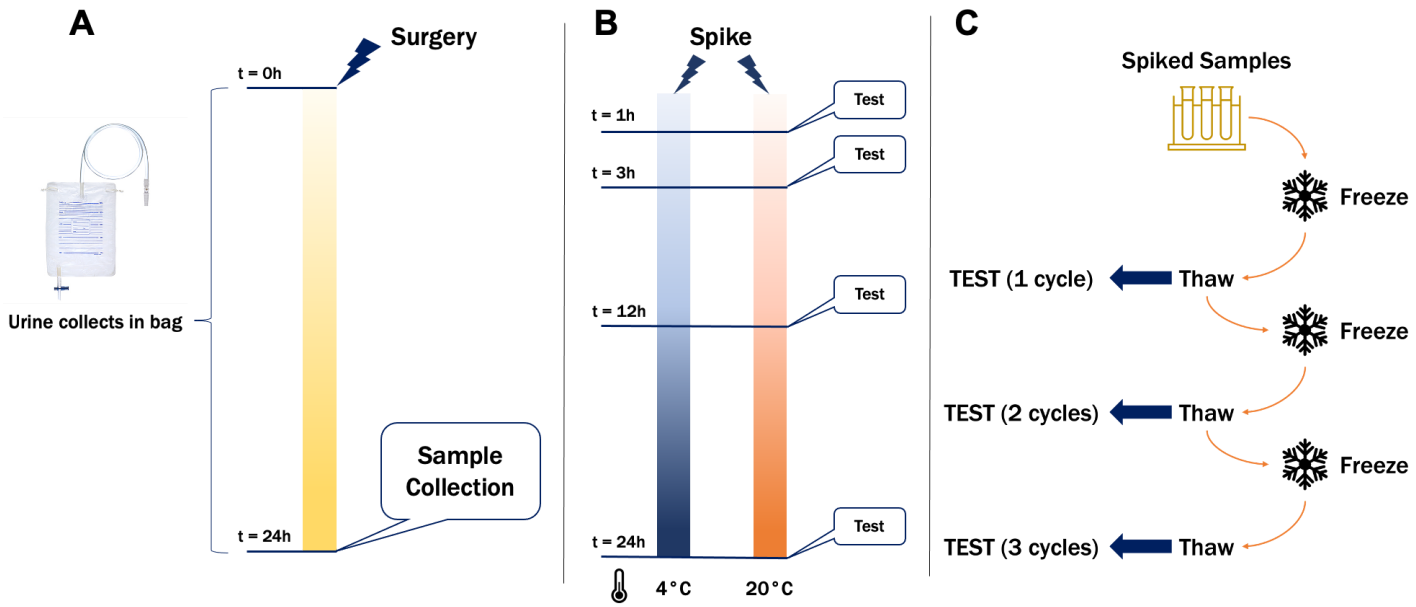

Figure S1. Schematic for collection of urine samples from cardiac surgery patients. Urine was continuously collected post-surgery, and the pooled sample was collected at 24 hours (A). Schematic for time and temperature experiment. Samples were spiked, then stored at either 4°C or 20°C for 1, 3, 12, or 24 hours before testing (B). Schematic for freeze-thaw experiment. Spiked samples underwent 1, 2, or 3 cycles before testing.