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Article

A Novel Multi-Biomarker Assay for Non-Invasive Quantitative Monitoring of Kidney Injury

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Abstract: The standard of care measures for kidney function, proteinuria, and serum creatinine (SCr) are poor predictors of early stage kidney disease. Measures that can detect chronic kidney disease in its earlier stages are needed to enable therapeutic intervention and reduce adverse outcomes of chronic kidney disease. We have developed the Kidney Injury Test (KIT) and a novel KIT Score based on the composite measurement and validation of multiple biomarkers across a unique set of 397 urine samples. The test is performed on urine samples that require no processing at the site of collection and without target sequencing or amplification. We sought to verify that the pre-defined KIT test, KIT Score, and clinical thresholds correlate with established chronic kidney disease (CKD) and may provide predictive information of early kidney injury status above and beyond proteinuria and renal function measurements alone. Statistical analyses across six DNA, protein, and metabolite markers were performed on a subset of residual spot urine samples with CKD that met assay performance quality controls from patients attending the clinical labs at the University of California, San Francisco (UCSF) as part of an ongoing IRB approved prospective study. Inclusion criteria included selection of patients with confirmed CKD and normal healthy controls; exclusion criteria included incomplete or missing information for sample classification, logistical delays in transport/processing of urine samples or low sample volume, and acute kidney injury. Multivariate logistic regression of kidney injury status and likelihood ratio statistics were used to assess the contribution of the KIT Score for prediction of kidney injury status and stage of CKD as well as assess the potential contribution of the KIT Score for detection of early stage CKD above and beyond traditional measures of renal function. Urine samples were processed by a proprietary immunoprobe for measuring cfDNA, methylated cfDNA, clusterin, CXCL10, total protein, and creatinine. The KIT Score and stratified KIT Score Risk Group (High versus Low) had a sensitivity and specificity for detection of kidney injury status (healthy or CKD) of 97.3% (95% CI: 94.6% - 99.3%) and 94.1% (95% CI: 82.3% - 100%). In addition, in patients with normal renal function [eGFR ≥ 90], the KIT Score clearly identifies those with predisposing risk factors for CKD, which could not be picked up by eGFR or proteinuria ($p<0.001$). The KIT Score uncovers a burden of kidney injury that may yet be incompletely recognized, opening the door for earlier detection, intervention and preservation of renal function.

Keywords: KIT Assay; chronic kidney disease; biomarker; non-invasive; urine; eGFR; cfDNA

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1. Introduction

Chronic kidney disease (CKD) is a worldwide public health problem. The major outcomes of CKD, regardless of cause, include progression to kidney failure, complications of decreased kidney function, and morbidity from cardiovascular disease. Increasing evidence indicates that some of these adverse outcomes can be prevented or delayed by early detection and treatment [1,2]. Despite a recognized high prevalence of early stages of CKD in the general population (approximately 11% of adults), early CKD is grossly underdiagnosed and undertreated, resulting in lost opportunities for renal preservation [3–7].

The Kidney Disease Outcomes Quality Initiative (KDOQI) of the National Kidney Foundation has defined a five-stage classification system of CKD in 2002, subsequently updated in 2012 [8]. The KDOQI recognizes that decreased renal function is associated with a wide range of complications, including hypertension, anemia, malnutrition, bone disease, neuropathy, and decreased quality of life, which can be prevented or ameliorated by treatment at earlier stages. Treatment can also slow the progression to kidney failure [9]. CKD is also a risk factor for adverse outcomes in other chronic diseases such as infections and cancer [10]. Thus, measures to prevent, detect, and treat CKD in its earlier stages could reduce the adverse outcomes of CKD.

Kidney biopsies are usually performed to better diagnose kidney injury, often based on a rise in the SCr, and thus can only detect established, often irreversible injury. The procedure is expensive, causes patient morbidity inclusive of bleeding complications, suffers from inter-operator variability, and may miss compartmentalized pathology leading to false negatives [11]. Kidney biopsies also cannot be utilized for serial sampling to monitor the progression of kidney injury.

Monitoring renal function with the SCr or the eGFR has value for established renal injury but has poor sensitivity for early CKD detection, as redundancy of renal reserve masks early injury. With these caveats in mind, KDOQI recommends routine urinary assessment of proteinuria for all eligible at-risk patients [2]. Unfortunately, proteinuria also generally detects established kidney injury and can be absent in some cases of advanced CKD [12]. Though the classification system of kidney disease, as defined by the Kidney Disease Outcomes Quality Initiative (KDOQI) of the National Kidney Foundation, has five stages, current clinical diagnosis, based on the SCr or proteinuria, largely captures stages 3 – 5 [eGFR < 60], whereas the earlier stages of kidney injury/disease at stages 1 – 2 [eGFR >60; 60-90 for stage 2; >90 for stage 1] usually go undiagnosed. Additionally, individuals can vary significantly in the rate and degree of damage that can accumulate over time with progressive kidney disease; thus, an ideal urine assay for kidney injury detection would need to be highly sensitive and quantitative.

Many urinary proteins and biochemical markers have been evaluated as noninvasive indicators of renal injury [13–20]. However, attempts to use them as general markers to screen patients for early renal injury and to identify the site of injury within the kidney have been disappointing. Additionally, many of these urinary markers have only been evaluated in acute kidney injury and acute ischemic tubular injury, with poor discrimination for CKD [15]. In fact, most available urine assays claim that they are unable to detect low levels of kidney injury.

The ***primary aim*** of this study was to perform an assessment of multiple novel and known urine biomarker measurements by simple, inexpensive technologies. Training and test data sets containing these biomarker measurements were subsequently used for generating a quantitative kidney injury score and prospectively assessing its utility for the detection of kidney injury with a high degree of sensitivity and specificity. Direct comparison to standard of care CKD assessment via eGFR and proteinuria was also performed using likelihood ratio tests. A ***secondary aim*** was to explore the potential utility of the test to distinguish subjects with normal eGFR but with predisposing risk factors for developing kidney damage and CKD. This report describes the process of biomarker selection, algorithm development and independent validation of the KIT Score as a novel diagnostic for the assessment and quantification of kidney damage.

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2. Experimental Section

2.1. Patient Selection

Residual, random spot urine was collected from 1169 sequential patients from the clinical labs at the University of California, San Francisco (UCSF) over 3 months, from October 2016-January 2017, as part of routine clinical testing at the Parnassus and Mission Bay campus clinical labs. Sample selection for a diagnosis of CKD was enriched by selecting urine samples obtained from the nephrology, diabetes, or cardiology clinics at UCSF. To facilitate statistical power, the study was enriched for patients at increased risk with kidney disease, using the following criteria: the patient had a confirmed diagnosis of CKD (ICD10 code N18 [21]), no diagnosis of CKD but a current diagnosis of diabetes, hypertension or auto-immune disease, no CKD but a positive family history of CKD, no CKD but age over 60, or from an ethnic minority status.

CKD was defined as an estimated glomerular filtration rate (eGFR) of ≤60 ml/min/1.73m^2 of body surface area persisting for ≥ 3 months and/or a prior diagnosis of a condition with known risk for kidney damage [22]. Kidney function was clinically assessed by the SCr and eGFR [calculated from the SCr and demographic variables from the electronic health record (EHR) [23]. For each patient, the presence and cause of kidney disease was confirmed through clinical review of medical and laboratory records, including evaluation of SCr, blood urea nitrogen, and proteinuria, as measured by either a 24-hour urine collection or a spot urine protein/creatinine ratio. Demographic information, any known cause of CKD, and SCr values within 3 months of the urine sample, were obtained from the EHR.

For purposes of KIT Score development, the relevant cause of CKD or the predisposing risk factor for CKD was captured from the EHR. The most common causes of CKD in this cohort were: immune-mediated systemic diseases that can cause renal injury (such as lupus nephritis, rheumatoid arthritis, Sjogren's syndrome), hypertension, diabetes (type 1 and type 2), glomerular disease (these cases were biopsy confirmed with IgA nephropathy, membranoproliferative glomerulonephritis or focal segmental glomerulosclerosis) and obstructive uropathy (neurogenic bladder, posterior urethral valves, hydronephrosis). In the CKD patient cohort, 74.8% of patients had 2 or more clinical diagnoses that would be relevant for CKD development and progression while 13.3% of patients had diabetes and hypertension as dual kidney injury risk factors in the absence of a clinical diagnosis of CKD, reflective of the high prevalence of these comorbidities in the general population.

The cause of CKD was not filtered and all contributing causes of CKD were captured. To avoid degradation of the biomarkers in the KIT assay, urine samples were either processed within an hour of collection or stored at 4°C and processed within 24 hours. Samples were discarded or not included for analysis in this study if the sample volume was less than 2 mL (minimum requirement for the KIT assay), if we were unable to confirm patient CKD diagnosis (no known urologic cause, no recent SCr measurements), or if the sample could not be processed within 24 hours from collection. Many samples faced the last exclusion criteria as these were residual urine samples from the UCSF Clinical lab and could not be send to the lab for the KIT assay within 24 hours due to logistical reasons. In addition, as the definition of CKD requires confirmation of the presence of abnormal kidney function that persists for 3 months or more; those urine samples for which only a single serum creatinine and urine protein determination were possible, were excluded from the analysis, to enable exclusion of patients with AKI or transient proteinuria of acute illness. After the above filtering process, we had a final selection of 343 unique urine samples from 343 patients. We also obtained additional urine samples from 54 healthy controls selected from volunteers who had good health, with normal SCr, no proteinuria, and no identifiable CKD risk factors.

The study adhered to the Declarations of Helsinki and Istanbul, was approved by the institutional review board of UCSF (IRB 16-21108), and requirement for informed consent was waived by the IRB.

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2.2. KIT Assay Methods

2.2.1. Sample Processing

Urine samples were collected in sterile containers (requested clinically to be collected as a clean catch and as a mid-stream void). There was no requirement for the urine sample to be collected at any specific time of the day. Samples were included even if they had micro- or macroscopic hematuria as this can be a condition of certain causes of kidney injury. Samples were included irrespective of presence or absence of proteinuria. Urine samples were centrifuged at 2000×g for 30 minutes at 4°C. The supernatant was separated from the urine pellet containing cells and cell debris. The pH of the supernatant was adjusted to 7.0 using Tris-HCl and stored at -80°C in the UCSF Biorepository until further analysis.

2.2.2. KIT Biomarkers

Based on extensive literature review and prior research studies, a small subset of biomarkers for inclusion in this study were selected to reflect different broad categories of causal injury pathways in the kidney, specifically immune-mediated. The KIT assay was designed to be a simple ELISA-based assay to perform, with low cost of goods, and deliberately avoids standard methods of cfDNA measurements such as sequencing [24], SNP quantification [25,26], or amplification [27]. KIT inputs normalized measurements of 6 primary urine biomarkers: cell-free DNA (cfDNA): as a measure of total apoptotic burden of kidney injury [28]; methylated cfDNA (m-cfDNA): as a marker to refine the proportion of cfDNA that may be more relevant to renal parenchymal injury [29,30]; CXCL10: as a marker of renal inflammation; clusterin: as a marker of renal tubular injury [31,32]; total protein: as a late marker of glomerular injury [33,34]; creatinine: as a normalizing marker for as it can be impacted by body mass, nutrition and hydration and utilized to avoid the need for a timed urine collection [35,36].

For ELISA-based measurement of cfDNA, we developed a proprietary 5′ biotinylated oligonucleotide complementary chemiluminescent immunoprobe to the ALU human element for the measurement of specific target cfDNA fragments, which we have found to be prevalent in the urine of patients with kidney injury [37]. Streptavidin-HRP (R&D Systems) and SuperSignal™ ELISA Femto Substrate (Thermo Fisher Scientific) were used for luminescent detection and quantitation. This approach overcomes the limitations of time-consuming sample processing, costly PCR amplification, and DNA sequencing methods employed otherwise to measure cfDNA in other biofluids [25,38]. In addition, the immunoprobe method allows for accurate detection of urine cfDNA without the need for amplification and without background assay noise from interfering substances commonly found in urine [39]. To enhance the interpretation of CKD injury based on the total cfDNA, we also assess the fraction of cfDNA that is methylated in urine by a proprietary assay (m-cfDNA). Total protein was measured using the Pierce™ Coomassie Plus (Bradford) Assay Kit (Thermo Fisher Scientific). CXCL10 and Clusterin were measured using custom generated human CXCL10 and Clusterin ELISAs. Creatinine was used to normalize the 5 biomarkers and was measured using the Creatinine Assay Kit (BioAssay Systems). Microwell plate readings were measured using a SpectraMax M2 Multi-Mode Microplate Reader (Molecular Devices). All assays were run in duplicates.

2.3. Statistical Analysis

2.3.1. KIT Score Development

For development and validation purposes, stratified random sampling was used to split the 397 patient cohort into training and test sets, stratified by kidney injury status. Specifically, 233 patient samples (n=37 healthy controls, n=196 confirmed CKD) from the overall cohort were randomly selected to train predictive models using statistical and machine learning methods. This information was used for development of the KIT Score algorithm, which integrates normalized urinary

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measurements for six selected DNA and protein biomarkers. In addition, we integrated the SCr and additional known risk variables for CKD such as race, gender and age for patients and healthy controls. Random forest modeling of these data reveals important relationships between total and m-cfDNA and the other markers for the detection of CKD with high sensitivity. Specifically, the KIT Score incorporates a multi-dimensional partition of these assay measurements based on identified clinical thresholds. A simple linear model incorporating the resulting partition was developed.

2.3.2. KIT Score Validation

The remaining independent subset of 164 patients (n=17 healthy controls and n=147 confirmed CKD) was subsequently used to prospectively validate the pre-specified KIT assay, KIT Score and clinical threshold for low and high risk of CKD. For this purpose, logistic regression was used to compare the (full) model with (log) protein/creatinine measurement, (log) eGFR measurement and the KIT Score versus the (reduced) model with (log) protein/creatinine measurement and (log) eGFR measurement alone. A P-value < 0.01 for the corresponding likelihood ratio test was considered significant. Similar logistic regression analyses were performed using the indicator variable for the categorical (Low and High) KIT Score Risk Groups based on the pre-specified clinical threshold obtained during development. The sensitivity and specificity of the resulting quantitative and qualitative KIT Score, along with 95% confidence intervals were calculated.

A secondary aim was to explore the potential utility of the KIT Score to distinguish subjects with early stages of CKD [eGFR 60-90, CKD Stage 2] as well as those with normal eGFR [> 90, CKD Stage 1] but with predisposing risk factors for developing kidney damage from healthy volunteers [eGFR > 90] who have no known predisposing risk factors. For this purpose, a t-test was used to compare the mean KIT Score for subjects with eGFR > 90 predisposed to CKD versus known healthy subjects. A p-value < 0.01 for the resulting t-test was considered significant. Additionally, logistic regression was used to model patient status (individuals predisposed to CKD versus healthy subjects) versus the pre-defined KIT Score. A p-value < 0.01 for corresponding likelihood ratio test for the KIT Score was considered significant.

3. Results

Of the 1169 patients recruited and urine samples collected, 201 samples met the exclusion criteria of <2 mL urine and were triaged from further analysis. The distribution of proteinuria is shown in the remaining cohort of 968 samples (Figure 1A) and ranged from 0 to 5469.73 mg/mmol with a median (IQR) of 4.69 (0-39.11) mg/mmol. The demographics of the 968 patients is shown in Table 1. Of the patients with overt injury, the etiologies of their kidney injury encompassed a broad and multiple range of diseases, with hypertension as a contributing cause for 42% of patients (Figure 1B). More than 60% of patients had more than one contributing cause to their kidney injury. Given that this is a reality for most CKD patients, particularly as they progress in later stages of CKD, the KIT Score was modelled to detect kidney injury irrespective of the underlying cause. As expected, patients with CKD had statistically significant lower eGFR ($P < 0.0001$), shown here as calculated using the MDRD equation [23] (Figure 1C). Despite proteinuria being the current gold standard for non-invasive assessment of kidney injury, there was no correlation between the eGFR and the urinary protein/creatinine ratio ($R^2 = 0.0087$), and proteinuria was poor at categorizing CKD patients into CKD stages (Figure 2). No significant differences were identified between the training and test cohorts in the final selection of 397 unique patients, selected based on clear phenotypes of healthy control (n = 54) and overt injury (n = 345).

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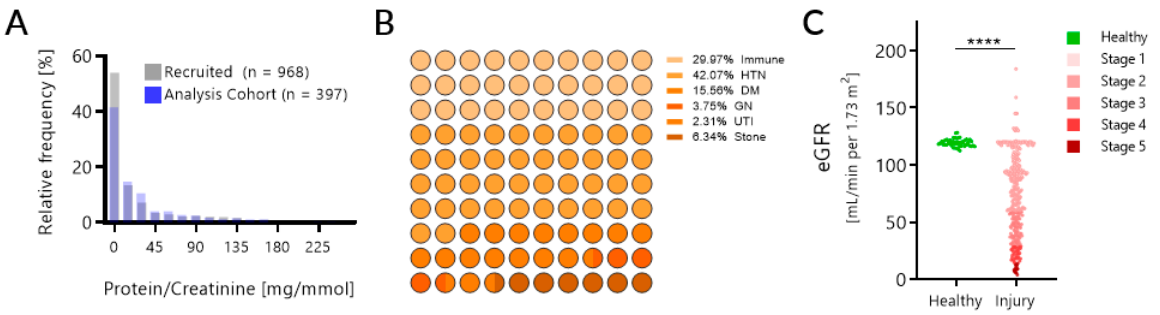

Figure 1. Cohort Characteristics. A. Proteinuria was assessed in the entire 968 patient samples and the distribution plotted in grey. The distribution for the 397 patients selected for further biomarker analysis is overlaid in blue. **B.** For the 343 patients out of the 397 with kidney injury, the contributing causes to CKD disease were plotted as a part of a whole plot, with the number indicating the proportion of patients with that aetiology. Immune injuries include causes such as immunological glomerulonephritis, lupus, and RA. GN injuries include causes such as minimal change and IgA nephropathy. **C.** The distribution of eGFR is depicted for the healthy and kidney injury subsets of 397 patients and color-coded by CKD stage.

Table 1. Demographics and Presenting Features of the study cohort.

Variable	Sampled Cohort (N = 397) Median (Range)	Training Cohort (N = 233) Median (Range)	Test Cohort (N = 164) Median (Range)
Age, years	53 (2 - 98)	53 (2 - 94)	52 (4 – 98)
Gender, women, %	49%	47%	53%
Race, % AA	13.2%	12.9%	13.7%
Proteinuria, [mg/mmol creatinine]	74.56 (0 – 8239)	76.58 (0 – 8239)	62.12 (0 - 3135)
Serum creatinine, [mg/dL]	0.98 (0.31 – 9.36)	1.06 (0.31 – 9.36)	0.83 (0.32 – 7.13)
eGFR [ml/min/1.73m²]	85 (4 – 184)	73 (4 – 159)	94 (6 – 184)
Cause of Kidney Injury, %			
• Immunological	30.0%	34.9%	22.5%
• Hypertension	42.1%	42.6%	41.3%
• Diabetes	15.6%	14.8%	16.7%
• GN	3.7%	1.0%	8.0%
• UTI	2.3%	1.9%	2.9%
• Kidney Stone	6.3%	4.8%	8.7%
Stage of CKD, %			
• Healthy	15.6%	15.3%	15.9%
• Stage 1	36.6%	28.2%	49.3%
• Stage 2	25.1%	26.8%	22.5%
• Stage 3	23.1%	26.3%	18.1%
• Stage 4	10.4%	12.4%	7.2%
• Stage 5	3.7%	5.3%	1.4%

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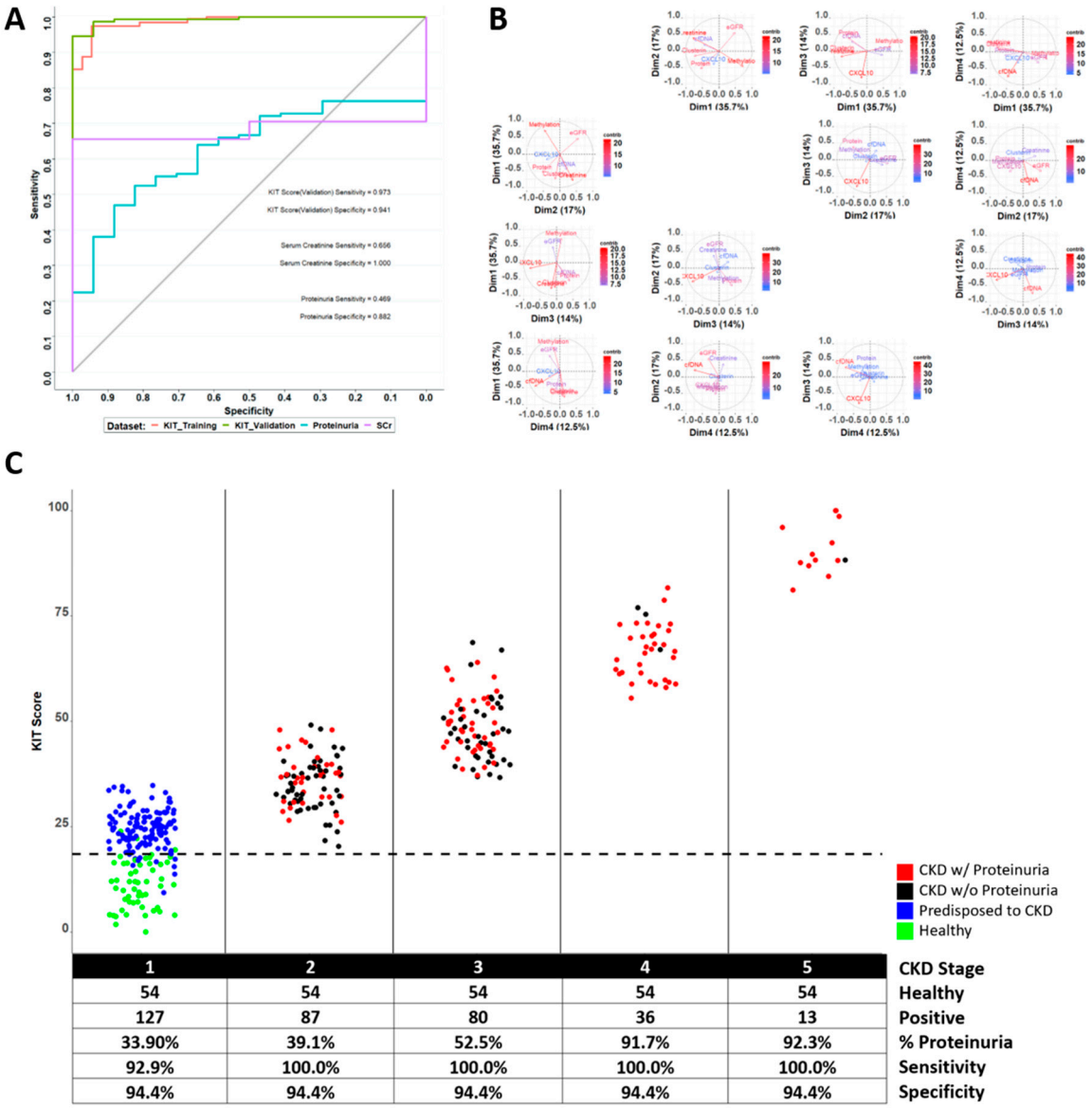
The primary aim of this study was to develop a composite KIT Score scaled from 0 (Low Risk) to 100 (High Risk) and prospectively assess the capability of a ***quantitative*** KIT Score for the detection of kidney injury with a high degree of sensitivity and specificity. Results of statistical analyses suggest that though current measures of renal function (as assessed by eGFR) and proteinuria are predictive of late stage kidney injury status (Table 2a), the quantitative KIT Score scaled from 0 (low risk) to 100 (high risk) provides predictive information of kidney injury status above and beyond proteinuria and renal function alone (likelihood ratio χ_1^2 = 52.6336, P-value <0.0001). Further, in this study, we can show that the application of the KIT Score outperforms assessment of CKD diagnosis and stage, over assessment by standard of care tests, proteinuria and renal function (Table 2b).



1	2	3	4	5	CKD Stage
54	54	54	54	54	Healthy
127	87	80	36	13	Positive
33.90%	39.1%	52.5%	91.7%	92.3%	% Proteinuria
92.9%	100.0%	100.0%	100.0%	100.0%	Sensitivity
94.4%	94.4%	94.4%	94.4%	94.4%	Specificity

Figure 2. Cohort Characteristics. Receiver Operating Characteristic Curves and Heat Maps for Kidney Injury. A. ROC curves for detection of kidney injury based on the KIT Score (training - orange and validation - green), serum creatinine – (purple) and protein/creatine (aqua). **B.** Principal Component Analysis Loadings Matrix for the KIT Assay shows independent contributions of different biomarkers for the KIT Score and variances in biomarker data explained by each principle component. **C.** KIT Score distribution as a function of CKD stage and proteinuria status (proteinuria positive if urine protein µg/mL/urine creatine µg /mL ≥ 0.2). The CKD stages are shown in blocks on the X Axis: Stage 1 CKD has no renal dysfunction and an eGFR of >90 ml/min/1.73m^2; stage 2 CKD corresponds to eGFR of 60-89 ml/min/1.73m^2; stage 3 CKD corresponded to moderate renal dysfunction, with an eGFR between 30-59 ml/min/1.73m^2. Patients with stage 4 CKD have severe renal

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dysfunction and an eGFR between 15-29 ml/min/1.73m². Stage 5 CKD, or end stage renal disease (ESRD), corresponds to an eGFR < 15 ml/min/1.73m² and generally leads to renal replacement therapy by dialysis or renal transplantation.

Regarding the ***qualitative*** KIT Score, a pre-defined clinical threshold of 18.5 was established in the training data for the KIT Score measurement of low and high risk of kidney injury. The resulting qualitative KIT Score Risk Group based on the clinical threshold of 18.5 (Low Risk Group ≤ 18.5, High-Risk Group >18.5) also provided predictive information of kidney injury status above and beyond proteinuria and renal function (likelihood ratio was χ_1^2 = 44.4650, P-value <0.0001). Consequently, the study met its pre-specified primary study endpoints, indicating that the algorithm incorporating the urine cfDNA, m-cfDNA, protein, and metabolite biomarkers are significantly associated with kidney injury status and may provide significant additional predictive information above and beyond typical measures used in CKD staging.

Table 2a. Multivariate Logistic Regression of Kidney Injury Status as assessed by SCr, eGFR and Proteinuria

Parameter	Estimate	df	s.e.	χ^2	P-Value
Intercept	44.1287	1	12.5600	12.3412	0.0004
eGFR	-8.8294	1	2.6193	11.3636	0.0007
Proteinuria	0.5019	1	0.2784	3.2508	0.0714

[1] Model Likelihood ratio χ_2^2 = 39.9369, P-value < 0.0001

Table 2b. Multivariate Logistic Regression of Kidney Injury Status as assessed by eGFR, Proteinuria and the KIT Score

Parameter	Estimate	df	s.e.	χ^2	P-Value
Intercept	-27.2922	1	29.7939	0.8391	0.3596
eGFR	3.5655	1	5.5538	0.4121	0.5209
Proteinuria	1.3181	1	0.8056	2.6765	0.1018
KIT Score	0.7874	1	0.2830	7.7395	0.0054

[1] Model Likelihood ratio χ_3^2 = 92.5704, P-value < 0.0001

Statistical analyses were performed to further assess the detection utility of the KIT Score for CKD. As shown by the receiver operating characteristic (ROC) curve in Figure 2A, the KIT Score derived from the test data has high sensitivity and specificity for detection of kidney injury. The estimated sensitivity and specificity of the KIT Score is 97.3% (bootstrap 95% CI: 94.6%, 99.3%) and 94.1% (bootstrap 95% CI: 82.3%, 100%), respectively. In contrast, the sensitivity and specificity of proteinuria was 46.9% (bootstrap 95% CI: 38.8%, 55.8%) and 88.2% (bootstrap 95% CI: 70.6%, 100%), respectively for the same samples. Similarly, the sensitivity of SCr was 65.6% (bootstrap 95% CI: 57.4%, 73.8%). As we recognize that the estimation of negative and positive predictive values of the KIT Score is dependent on the prevalence rate of the disease, we use the prevalence rate of hypertension as an example. The prevalence of hypertension in the general US population is approximately 33%. Consequently, the estimated positive and negative predictive value of the quantitative KIT Score for hypertension would be ~ 89.1% and 98.2%, respectively.

Statistical analyses were performed to further assess the contribution of individual biomarkers to the KIT Score. Table 2c provides results of multivariate logistic regression analyses of kidney injury status as a function of the individual biomarkers from the combined data. The biomarkers of (log) cfDNA (P-value = 0.0052), m-cfDNA (P-value < 0.0001), protein (P-value < 0.0001), and CXCL10 (P-value 0.0302), were all significant predictors of kidney injury status. Clusterin was the only kidney

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injury status biomarker that was not significant in multivariate analyses (P-value = 0.1671). Urine creatinine is used for normalization purposes in the KIT algorithm, and controls for diurnal and hydration variations (thus obviating the need for any timed urine sampling) and was also not significant in multivariate analyses (P-value = 0.2506). Furthermore, the correlations among the individual biomarkers were quite small (largest correlation $R^2 = 0.14$ was between eGFR and protein), suggesting that each of the biomarkers is providing independent information towards the prediction of kidney injury status.

Table 2c. Multivariate Logistic Regression of Kidney Injury Status as assessed by Individual KIT Urine Biomarkers

Parameter	Estimate	df	s.e.	χ^2	P-Value
Intercept	56.0716	1	12.9866	18.6451	<0.0001
eGFR	-12.5302	1	2.7181	21.2521	<0.0001
Urine cfDNA	-0.2720	1	0.0973	7.8120	0.0052
Urine m-cfDNA	-1.1260	1	0.2655	17.9946	<0.0001
Urine Protein	0.7976	1	0.1815	19.3702	<0.0001
Urine CXCL10	1.1304	1	0.5216	4.6959	0.0302
Urine Clusterin	-0.3506	1	0.2538	1.9099	0.1671
Urine Creatinine	0.6448	1	0.5613	1.3202	0.2506

Principal component analyses (PCA) further elucidate the relationship of independent linear combinations of the biomarkers to the total variability in assay measurements. Figure 2B provides the resulting PCA loadings for the first four principal components, which account for approximately 80% of the total variance in the biomarker data. The loadings for the first and second principle components are dominated by differences in renal function (shown here as the eGFR) and DNA methylation versus the remaining biomarkers. In contrast, the loadings for principal components 3 and 4 are dominated almost entirely by CXCL10 and cfDNA, respectively. These results suggest that CXCL10 and cfDNA are providing additional individual predictive information from the remaining factors. Evaluation of abundance of cfDNA or m-cfDNA revealed that levels of both biomarkers in urine was highly variable across the different categories of renal disease and did not correlate with the stage of CKD. As stated earlier, a similar distribution is also seen for proteinuria alone. These factors highlight the importance of including the 6 biomarkers and developing the composite KIT score for CKD assessment.

CXCL10 is a key inflammatory cytokine, dysregulated in immune-mediated renal injury [40,41]. Studies from our group on alloimmune kidney inflammation [42–45] and from other groups on kidney inflammation in autoimmune diseases [46–48], confirm that the urine is a mirror for intra-renal molecular events and high renal CXCL10 is very accurately reflected by high urine CXCL10 mRNA [49,50] and CXCL10 protein levels [43,51,52]. To evaluate if CXCL10 identifies a specific cause of CKD, such as immune mediated causes of renal injury from systemic lupus erythematosus, rheumatoid arthritis, ANCA positive vasculitis (selected in our cohort), we evaluated which CKD categories had high abundance of urinary CXCL10. Approximately 30% of the immune-mediated cohort had very high CXCL10 values (>100 pg/ml); whereas the remainder had low/undetectable CXCL10. As these are random urine samples it is possible that many patients in this cohort have quiescence of their immune-mediated disease, as most patients were on maintenance immunosuppressants. To our surprise, ~30% of the hypertensive and diabetic cohorts also had very high urinary CXCL10 levels. None of these patients were on immunosuppressive drugs or were

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known to have any systemic immune-mediated disease. Additionally, there was no association of urinary CXCL10 levels and CKD stage.

Figure 2C displays the KIT Score as a function of CKD stage as defined by accepted eGFR thresholds in the full cohort of 397 patient samples. The healthy controls are marked in green and are shown in a pre-CKD1 or CKD 0 stage where the eGFR is >90 ml/min/1.73m^2 like in CKD stage 1, but there is no proteinuria or identified risk for kidney injury. It is important to note that ~15% of the healthy controls tested have a mild elevation in their KIT Score to just above the risk threshold of 18.5. As none of the KIT Score results were disclosed to the healthy controls, we have no way of assessing if indeed more subtle risk factors or early kidney injury could have been identified on more thorough clinical and laboratory evaluation of controls with KIT Scores >18.5.

Figure 2C also shows the distribution of the quantitative KIT Score in all patients across different CKD stages. Patients with proteinuria are marked, with presence or absence of proteinuria being a binary variable (black dots= positive for proteinuria, using a threshold cutoff of urine protein/creatinine ratio of >0.2). We see that many patients in CKD stage 1, with "normal" renal function, also have no proteinuria (60%) and would thus be assessed by current SOC testing to not have any active kidney injury. Table 3 shows a breakdown of the mean KIT Score by CKD stage, and we note a significant trajectory of increasing KIT Score by advancing CKD stage, as expected. Importantly, the KIT Score identifies 92% of patients in CKD stage 1 who have no proteinuria as having early kidney injury. In CKD stage 2-3, where again 60% of patients have no proteinuria, the KIT Score picks up all patients as high-risk and quantitates their kidney injury status. In CKD stages 4-5, proteinuria and the KIT Scores are more concordant as kidney damage is advanced and proteinuria is a late marker of renal injury. These results clearly demonstrate that, unlike the SOC tests, proteinuria, SCr and eGFR measurement alone, the KIT Score can accurately identify a large cohort of kidney injury patients with early stages of CKD. The assay thus achieves its primary objective of predicting kidney injury earlier than current SOC.

Table 3. Distribution of Mean KIT Scores and presence/absence of proteinuria by CKD Stage.

CKD Stage by eGFR	Mean KIT score/ CKD stage	% Patients w/o Proteinuria	% Patients w/o Proteinuria who have High-Risk for Kidney Injury (KIT Score >18.5)
CKD Stage 1	24.4	66% (84/127)	91% (77/84)
CKD Stage 2	35.3	60% (53/87)	100%
CKD Stage 3	48.4	47% (38/80)	100%
CKD Stage 4	66.9	8% (3/36)	100%
CKD Stage 5	90.8	7% (1/13)	100%

[1] Proteinuria becomes more predictive for kidney injury with advancing CKD stages and progression of kidney injury. Recognized as a late marker of kidney injury, proteinuria becomes more invariant in CKD stage 4/5. In earlier stages of CKD, the KIT Score detects kidney injury, independent of proteinuria.

To address the study's secondary aim, we find that the KIT Score was able to distinguish subjects with early stages of CKD [eGFR > 60, CKD Stage 1 and 2] as well as those with normal eGFR [> 90, CKD Stage 1] but with predisposing risk factors for developing kidney damage from truly healthy volunteers [eGFR > 90] without known risk factors. The mean KIT Score for the healthy volunteers with no risk factors of CKD was 11.0 (95% CI: 9.5, 12.6). In contrast, the mean KIT Score for subjects with early stages of CKD [eGFR > 60, CKD Stage 1 and 2] and for patients with normal eGFR [> 90, CKD Stage 1] but with predisposing risk factors for developing kidney damage were 28.9 (95% CI: 27.9, 29.9) and 24.4 (95% CI: 23.6, 25.3), respectively. The resulting t-tests were highly significant (p-

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values < 0.00001) indicating a significant difference in mean KIT Scores from the healthy volunteers. Logistic regression analyses and associated likelihood ratio tests also reveal a strong association between the KIT Score and predisposition to kidney injury in those subjects with eGFR > 90. Within those subjects with eGFR > 90, logistic regression resulted in a likelihood ratio $\chi_1^2 = 148.4$ (LR p-value < 0.0001) and a sensitivity and specificity of 92.9% (exact 95% CI: 87.0%, 96.7%) and 94.4% (exact 95% CI: 84.6%, 98.8%), respectively, for detecting individuals predisposed to CKD at the pre-defined clinical threshold of 18.5 for the KIT Score. In subjects with early stages of CKD [eGFR > 60, CKD Stage 1 and 2], similar analyses resulted in a likelihood ratio $\chi_1^2 = 196.5$ (LR p-value < 0.0001) and a sensitivity of 95.8% (exact 95% CI: 92.2%, 98.1%). These results suggest that the KIT Score can potentially detect early stages of CKD with a high degree of accuracy, irrespective of their renal function or the presence of proteinuria.

4. Discussion

The driving force behind the development of the KIT assay and KIT Score was a recognition of the massive, and increasing, burden of unrecognized kidney injury [53], compounded by the weakness of traditional standard of care metrics [54]. Tracking kidney damage in individuals by urine sampling has always been a highly desirable goal, as the biological signal from urine can provide a highly informative window into the health of the kidney. Nevertheless, a urine test to predict kidney injury, irrespective of underlying cause, remains a clinical unmet need and a biochemistry challenge to execute, as there are many interfering substances in urine such as proteases [55,56], resulting in biomarker degradation, and high inter-individual variance in urine pH, all of which can confound the development of a robust laboratory assay. Targeted proteomic studies, fuelled by protocolized urine sampling from prospective clinical trials, spanning a decade (in our laboratories at Stanford University and UCSF), has led to the development of standard operating procedures (SOP) for urine collection, stabilization, processing, evaluation of interfering substances, preservation and transport from distant sites to a central processing lab [57]. In addition, over a decade of transcriptional and LC-MS/MS based urine proteomic studies [44,51,52,58–61] have resulted in deeper biological understanding of kidney injury, across different causes of CKD, which drove the selection of biomarkers for inclusion in the KIT assay, to represent injury across different intra-renal compartments.

This comprehensive search led us to six biomarkers: cfDNA, methylated cfDNA, clusterin, CXCL10, creatinine, and urinary protein. Cell-free DNA has been recognized as a sensitive marker of disease burden in the plasma of patients with autoimmune diseases [62,63] and with tumours [64]. However, their utility in the plasma is limited in the setting of multiple diseases and morbidities, as total cfDNA burden would reflect the cumulative processes of various diseases while organ or site-specific cell-free DNA measurement in the plasma requires advanced sequencing technologies and bioinformatics [65,66]. However, because cfDNA in the urine specifically reflects contributions from the kidney, the KIT cfDNA assay enables extremely sensitive detection of kidney injury via an inexpensive ELISA-based assay. Measurement of methylated fragments of cfDNA provides additional specificity regarding the type of injury. While global hypermethylation has been associated with immune-related kidney injury and increased fibrosis, global hypomethylation is associated with aging-related renal decline and renal ischemia-reperfusion injury [29,30,67]. Like our rationale in the measurement of cfDNA, we find that global changes in the methylation state of the cfDNA enable accurate discrimination between kidney disease states without the need for loci-specific sequencing or PCR.

CXCL10 has been well established to be a marker of immune-mediated injury in a variety of contexts due to its role as a ligand for the CXCR3 receptor [40,50,68–71]. We have previously show that CXCL10 and cfDNA as measured via the KIT Assay can detect chronic lung allograft dysfunction in lung transplantation as well as rejection in kidney transplantation [72–74]. Strikingly, what we found in the present study is that there is a significant number of patients with traditionally non-immune kidney diseases, such as hypertension and type 2 diabetes, that had elevated CXCL10, potentially indicating a broader utility in the detection of early stage kidney injury. Prior studies have

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identified type 2 diabetes to have a significant CXCL10-mediated component [75] and have identified endothelial-cell produced CXCL10 as a contributor to essential hypertension [76]. Our findings suggest that CXCL10 can identify not only patients with immune-mediated kidney injury, but also those of other causes, ones that tend to present insidiously with late clinical symptoms relative to disease progression.

Urine total protein and clusterin are well-established markers of kidney dysfunction [31,33,34] and creatinine is well-validated in the field to serve as a normalizing biomarker [35,36]. Surprisingly, we find that clusterin, after multivariate analyses, is not significant in our model of kidney injury in the context of our other biomarkers. This may be due to a number of causes, including its correlation to the total protein to creatinine ratio, the components of which are already included in the model [31], as well as the high spot variation due to the ultradian rhythms of the tightly correlated plasma and urinary levels of clusterin [77,78].

Early detection of kidney injury has very important ramifications on limiting the rate of CKD progression and positively impacting health care world-wide. As renal hemodynamic changes are acutely sensitive to systemic perturbations, improved management of the main underlying causes of CKD would be an immediate benefit, specifically improved and tight control of blood pressure [79], stability and tight control of hyperglycaemia [80], and early detection and prompt immunomodulation for abrogation of renal inflammation and injury in immune mediated systemic diseases such as systemic lupus erythematosus [81] and rheumatoid arthritis [82]. Persistent and early elevations of the score could trigger renal imaging to evaluate for obstructive uropathy, with prompt intervention to prevent high intra-renal pressure and progressive renal interstitial fibrosis and tubular drop out. Early detection of renal injury is most likely going to occur in the primary care setting, where the availability of a rapid throughput, simple assay with a quantitative kidney risk score read-out can trigger earlier referral to a nephrologist for blood pressure control, dietary modification, treatment of coronary and/or peripheral vascular disease, the underlying cause of the CKD and the consideration of renal preserving therapies [83,84]. The ability to quantitatively track the resolution of the KIT Score over time provides an opportunity to track kidney injury resolution. In addition to choices to use medications or support to better control the systemic disease, new reno-protective drugs, such as the SGLT2 inhibitors and others [85], further highlight how crucial it is to be able to detect very early kidney injury, treat and reverse it.

Delays in kidney injury detection and consequently patient referral is a significant obstacle to getting new patients into effective treatment regimens while they still have a chance to maximize the benefits of renal preserving therapies. To emphasize how many patients “miss” the opportunity for diagnosis of CKD in earlier stages of their disease, as patients with ESRD use the emergency department at a rate six times higher than the national mean rate for US adults, half of which result in hospital admission [86]. Thus, in addition to using the KIT Score to track renal recovery, there is tremendous value to also using this assay to track renal injury progression so that interventions can be brought in as needed to stabilize KIT Score trajectory progression. The observation that a third of patients with CKD aetiology due to hypertension or diabetes also have a strong immune/inflammatory milieu as part of their renal injury, suggest that appreciation of the biological heterogeneity of different categorical diseases by the individual biomarker values in the KIT assay, and other interrogative studies, will allow for more customized approaches to treatment for CKD patients.

Ongoing studies are planned and underway whereby serial assessment of the pre-defined KIT Score with the pre-defined KIT assay will allow us to assess the value of individual biomarkers in the assay during renal injury and renal recovery. In addition, we are setting up collaborations with pharmaceutical partners where serial assessment of the KIT assay can be used as a means to non-invasively and accurately track for early kidney injury from drug nephrotoxicity, inherent to many immune-modulators, such as calcineurin inhibitors [87] and anti-TNF agents [88]; chemotherapeutic agents such as cisplatin [89], aminoglycosides [90], and newer immunotherapies [91], as well as exposure to radionuclide contrast media for imaging purposes [92]. Additional cohorts are being assembled for analysis where patients have been followed up longitudinally over the course of CKD

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progression, which will allow us to better understand the granular trajectory of the KIT Score and possibly help refine CKD staging.

As the assay biomarkers were chosen and the assay developed with the specific intent to pick up very early kidney injury, we also observe that the KIT assay can detect that a small percentage of "normal" controls display urine KIT Scores that hover at the high-risk threshold of 18.5. Though not confirmed, it is possible that this is not assay noise and these cases are true positives with very early identification of kidney injury risk in the pre-CKD 1 or CKD 0 stage. As 96% of people with CKD do not realize they have it [93], one significant challenge in developing kidney injury models is the high likelihood that patients recruited as healthy controls actually have early stage kidney disease. For example, the human longevity project, led by Craig Venter with the Health Nucleus test, extensively sequenced and performed additional screening tests of symptom-free adults and found clinical correlates of potential disease in 21% of their "healthy" study participants inclusive of urologic/renal diseases, suggesting the need for increased screening. This is especially true of diseases where early intervention can delay or even reverse disease progression. We find CKD and kidney injury as a whole to be an exemplary disease in which this is true, as numerous lifestyle and therapeutic interventions can prevent further progression of kidney function decline [9,83,84,93].

A limitation of the current study design is the use of a population enriched for CKD subjects from a tertiary care site. Subjects obtained from more screening and community settings may be more representative of the general population, particularly for the detection of early stage CKD. Additionally, the study was cross-sectional and may not have fully represented early and late stage CKD. Longitudinal studies monitoring patients with signs of early stage CKD are planned for improved assessment of the early detection capabilities of the KIT Score and for the impact of early detection on CKD progression. Finally, it may be possible to further augment the KIT Score with additional biomarkers to allow improved differential diagnosis of CKD and minimize the need for serum creatinine and proteinuria measurement.

The positive economic impact of early kidney injury detection and its treatment cannot be underscored. Almost a third of the Medicare budget is devoted to the management of kidney injury and disease in the US. The loss of kidney function in CKD stage 5 adds an additional fiscal burden of ~$80,000 per year due to dialysis support. Although renal transplantation enables patients to come off dialysis, the shortage of renal donors, both living and cadaveric, renal transplantation, with an initial procedure cost of ~$100,000, followed by maintenance medication costs of ~$20,000/year, results in only a small dent in the dialysis Medicare budget [94]. The trajectory for the numbers of patients with CKD is expected to continue to rise world-wide, with greater trends in obesity, resulting in rising numbers of people with hypertension and diabetes [95]. In addition, ethnic variations in renal diseases, drive national health care problems, requiring population screening with kidney biopsies for early detection of renal injury from IgA kidney disease in South-East Asia [96], where IgA kidney disease is the prime cause of renal failure. The inclusion of a sensitive non-invasive assay for renal injury, to replace invasive, high-cost, high-morbidity biopsy procedures, would result in major socio-economic benefits for these at-risk populations.

In conclusion, this study provides the blueprint for the KIT assay biomarkers, the KIT assay algorithm development process, the KIT Score definition and its performance for the early detection of kidney injury, and its direct comparison with current standard of care tests. Further studies are needed, and planned, for longitudinal screening of patients over time, to better understand the natural history of progression of CKD, the benefits of early detection, intervention and monitoring, and the infection points where CKD injury becomes fixed and progressive.

Author Contributions: M.S., J.Y., D.W., and T.S. designed the study and experiments; J.L., I.D., D.L., K.S., R.S., A.C., T.T.S., M.L., carried out experiments; D.W., J.Y., R.S., and M.S. analyzed the data; D.W., J.Y., and M.S. made the figures; J.Y., D.W., R.S., T.S., E.S. and M.S. drafted and revised the paper; all authors revised the manuscript critically for important intellectual content and approved the final version of the manuscript. M.S. had full access to all the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis.

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Conflicts of Interest: M.M.S., D.W., and J.Y.C.Y. are founders of KIT Bio, Inc. (Los Altos, CA), IP for which is exclusively owned by the Regents, University of California San Francisco and licensed to KIT Bio. All other authors declare no conflict of interest.

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