PRECISION IN EVERY PROTEIN

CASE STUDY

Leveraging FibroKey™: Precision Multiplex Monitoring of Fibrosis Biomarkers in Chronic Kidney Disease for Tracking Disease Progression and Therapeutic Response

Introduction

Chronic Kidney Disease (CKD) affects millions worldwide and is a leading cause of morbidity and mortality. Early detection of fibrotic processes is crucial for developing therapeutic strategies to mitigate kidney function loss. FibroKey™ offers a robust platform for monitoring fibrosis biomarkers quantitatively, particularly proteins involved in extracellular matrix (ECM) remodeling and inflammation. Using Mass Spectrometry (MS) multiplexed assays, FibroKey™ enables pharmaceutical companies to track disease progression and therapeutic effects in urine samples, providing valuable insights into fibrosis pathways and CKD progression.

Study Design and Method Overview

The study employed a patient cohort of 131 individuals divided into five groups based on CKD severity, ranging from early to late stages. Urine samples were collected and normalized against creatinine and total protein levels to adjust for urine concentration variability. Heavy-labeled protein and peptide standards were used when available to quantify key biomarkers, ensuring robust measurement of fibrosis-related proteins across all CKD stages. The laboratory work spanned one week, with two days dedicated to LC-MS analysis utilizing validated methodologies. InoKey™, Inoviv's proprietary platform, was instrumental in validating the results and analyzing the biomarker data to ensure reproducibility and clinical relevance.

CKD Staging and Patient Cohort

Patients were classified according to CKD stages using eGFR, a widely accepted measure of kidney function. The staging was as follows:

- **CKD Stage 1**: eGFR > 90
- **CKD Stage 2**: eGFR 60-89
- **CKD Stage 3**: eGFR 30-59
- **CKD Stage 4**: eGFR 15-29
- **CKD Stage 5**: eGFR < 15

Statistical analysis included Kendall's Tau correlation and ordinal logistic regression, identifying a strong correlation between CKD stage severity and key biomarkers such as C3, MMP7, and LGALS3.

Biomarker Selection and Validation

FibroKey™ measured 33 biomarkers linked to fibrosis and inflammation, selected based on their disease relevance and biomarker function in CKD progression. While approximately 22 markers are reliably detectable in healthy populations, the additional markers serve a strategic purpose: they provide deeper insights for disease-state applications, capturing proteins that may become more relevant or measurable as disease progresses. This approach ensures the assay's utility across both standard and disease-specific studies. Among the key markers detected were:

Key Markers Detected in FibroKey™	
Galectin-3 (LGALS3)	A marker of fibrosis and inflammation, elevated in CKD stages 3-5.
Matrilysin (MMP7)	Associated with ECM remodeling and increased in later CKD stages.
Annexin A1 (ANXA1)	Linked to inflammation, significantly upregulated in advanced fibrosis.

Method Overview

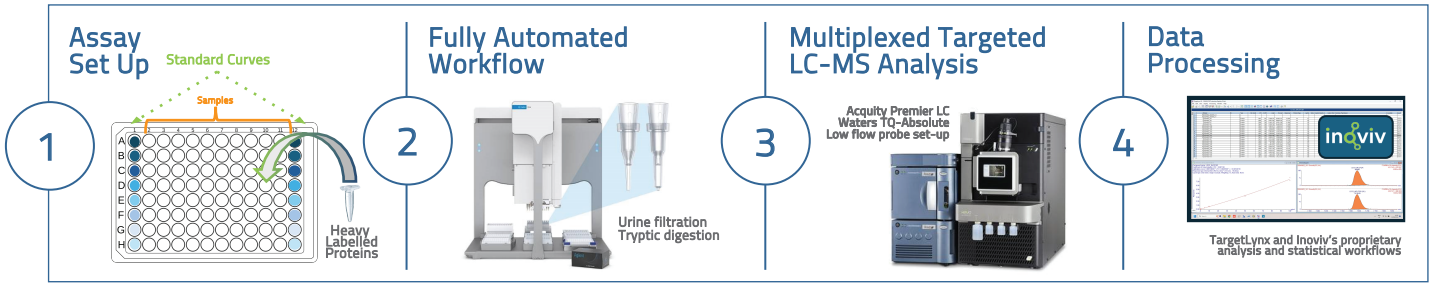


Figure 1. Visual Representation of the FibroKey™ Assay. 500µL of urine were filtered through protein desalting plates on an Assaymap Bravo, Agilent. The concentrated proteins were then reduced, alkylated, and digested using trypsin on the same automated platform. When available, heavy-labeled protein standards (n=14) were spiked prior to urine filtration. Alternatively, heavy-labeled peptides containing a tryptic tag were spiked prior to enzymatic digestion. Tryptic peptides were quantified using a Xevo TQ Absolute triple quadrupole mass spectrometer coupled to an ACQUITY Premier LC fitted with an ACQUITY HSS T3 Column (1.6µm, 1mm x 150mm) at a flow rate of 100 µL/min and a total run time of 15 minutes per sample. Unique peptides and corresponding stable isotope-labeled internal standards were monitored for each target protein using a scheduled MRM method and at least 2 MRM transitions per peptide. Chromatograms were processed using TargetLynx software. The most intense transition for each peptide was used for absolute quantification. Analytical validation and statistical analysis were performed using Inoviv's proprietary workflows. A creatinine assay and a Total protein assay are also run in each patient sample to allow data normalization.

Demographic and Clinical Characteristics of the Study Cohort Across CKD Stages

Characteristic	N	1 N=27¹	2 N=29¹	3A N=18¹	3B N=17¹	4 N=29¹	5 N=11¹	p-value²
Age	131	44 (29, 55)	51 (41, 62)	58 (41, 67)	57 (38, 65)	61 (43, 73)	68 (44, 83)	0.007
Sex	131							0.047
Female		15 (56%)	17 (59%)	3 (17%)	7 (41%)	17 (59%)	4 (36%)	
Male		12 (44%)	12 (41%)	15 (83%)	10 (59%)	12 (41%)	7 (64%)	
eGFR	131	104 (96, 114)	78 (68, 85)	53 (51, 56)	38 (36, 40)	22 (19, 27)	8 (5, 14)	<0.001

Figure 2. The table summarizes the distribution of age, sex, and estimated glomerular filtration rate (eGFR) across CKD stages 1 to 5. Age and eGFR values are presented as medians with interquartile ranges (Q1, Q3), and sex distribution is shown as the number and percentage of females and males in each stage¹. The p-values represent the results of the Kruskal-Wallis rank sum test for age and eGFR and the Pearson's Chi-squared test for sex distribution, comparing differences across CKD stages. Significant differences were observed in age ($p = 0.007$) and eGFR ($p < 0.001$), while the distribution of sex approached significance ($p = 0.047$)².

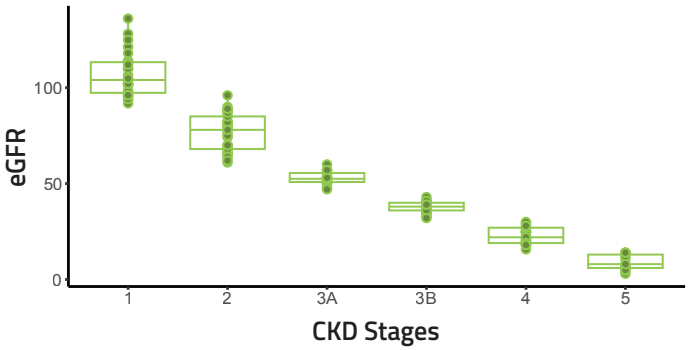


Figure 3. CKD Stages Based on Estimated Glomerular Filtration Rate (eGFR). This boxplot illustrates the distribution of estimated glomerular filtration rate (eGFR) values for individual patients across different CKD stages. eGFR is used to classify CKD stages, with lower eGFR indicating more severe kidney disease. Each dot represents the eGFR value for a single patient, while the boxplot summarizes the distribution for each stage. The box represents the interquartile range (IQR), with the horizontal line indicating the median eGFR value for that stage.

Biomarker Selection and Validation

The biomarkers measured were validated using heavy-labeled recombinant proteins, allowing for accurate quantification and reproducibility across samples. The InoKey™ workflow ensured precise assessment, validation, and analysis of the data, making the FibroKey™ assay a reliable tool for monitoring fibrosis progression.

Results

The results showed a clear correlation between CKD stage severity and the expression of key fibrosis markers. For example, LGALS3 levels were significantly elevated in CKD stages 3-5, reflecting increased fibrosis. MMP7, involved in ECM breakdown, showed increased levels as CKD progressed, making it a potential marker for late-stage fibrosis.

Statistical analysis using ordinal logistic regression confirmed significant associations between CKD stages and the levels of fibrosis markers. Among the biomarkers, Cystatin C was detected across all CKD stages, with elevated levels correlating to worsening kidney function. The total protein normalization approach provided a more consistent measure, addressing the variability in creatinine levels typically seen in CKD patients.

Statistical Analysis

Statistical tests, including the Kruskal-Wallis test, ordinal logistic regression, and Kendall's Tau correlation, were applied to assess relationships between biomarker levels and CKD severity. Key findings included significant changes in biomarkers such as MMP7 and C3, which were validated as strong indicators of fibrosis progression across different CKD stages.

To further validate the results, Inoviv's standard protocol was followed, including key performance metrics such as limit of quantification (LOQ), limit of detection (LOD), and evaluation of inter-day and intra-day variability. Each of these metrics adhered to the maximum allowable 20% variation in both precision and accuracy, providing robust, regulatory-compliant results.

The data analysis confirmed significant associations between the concentration of these fibrosis biomarkers and CKD progression. These findings not only reinforced the validity of FibroKey™ but also highlighted its potential utility in therapeutic decision-making processes and disease monitoring.

Monitoring Disease Progression

FibroKey™ offers an effective, non-invasive solution for monitoring CKD progression in subject cohorts. By analyzing urine samples, the assay tracks fibrosis-related biomarkers, providing insights into the severity and progression of kidney disease. Notably, Cystatin C and ICAM1 were highly correlated with CKD severity, indicating their potential as biomarkers for disease staging and therapeutic response.

The assay detected 22 biomarkers, including 17 with absolute quantification and five with relative quantification. Designed to provide comprehensive coverage, FibroKey™ quantifies key markers both at endogenous levels and specific to disease states, enabling robust tracking of CKD progression across various stages. This depth of insight supports clinical trials and therapeutic development, adhering to FDA bioanalytical guidelines for reliability in research and clinical settings.

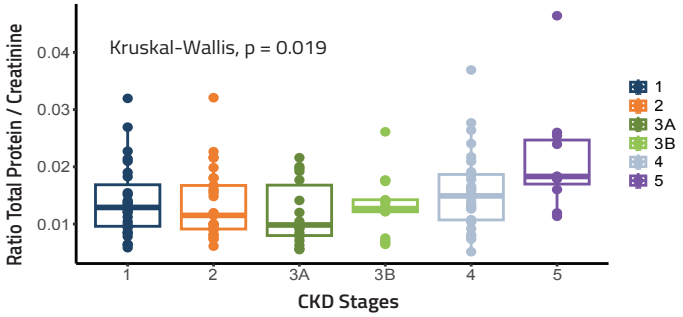
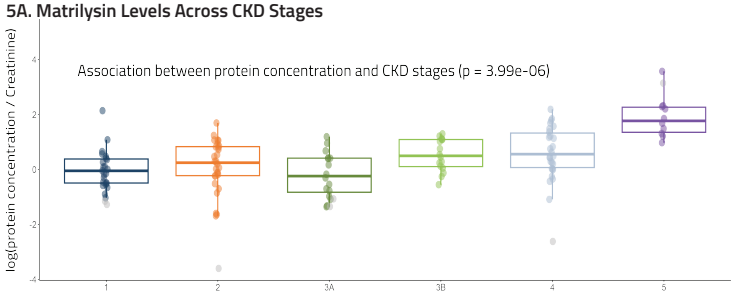
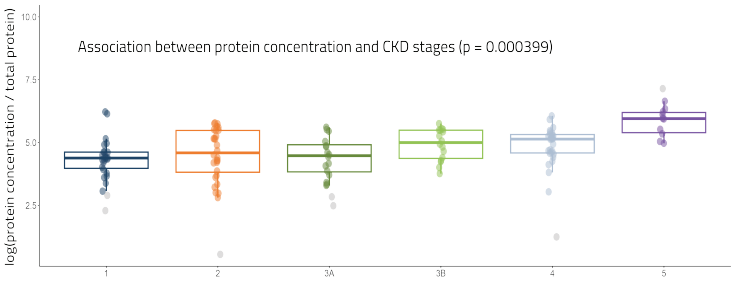


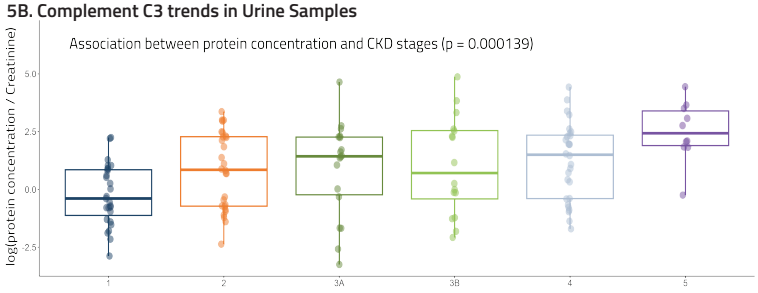
Figure 4. Statistical Correlation Across CKD Stages. CKD Stages Based on Estimated Glomerular Filtration Rate (eGFR). This boxplot illustrates the distribution of estimated glomerular filtration rate (eGFR) values for individual patients across different CKD stages. eGFR is used to classify CKD stages, with lower eGFR indicating more severe kidney disease. Each dot represents the eGFR value for a single patient, while the boxplot summarizes the distribution for each stage. The box represents the interquartile range (IQR), with the horizontal line indicating the median eGFR value for that stage.

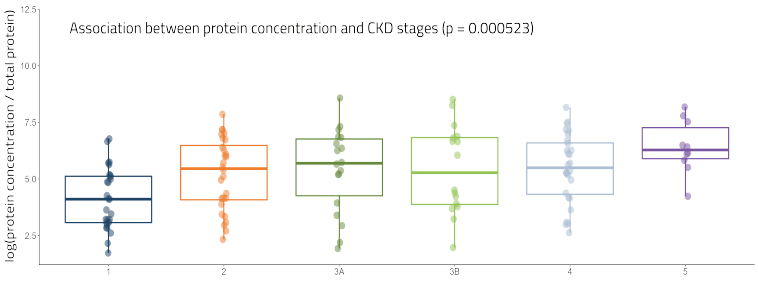
5A. Matrilysin Levels Across CKD Stages





5B. Complement C3 trends in Urine Samples





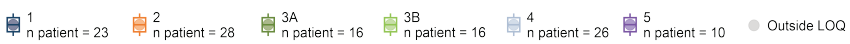


Figure 5. These boxplots represent the log-transformed concentrations of a protein normalized to creatinine (top panel) and total protein (bottom panel) across different CKD stages. Each dot represents an individual patient's protein concentration value, and the boxplots summarize the distribution for each stage. The boxes represent the interquartile range (IQR), with the horizontal line indicating the median. The number of patients in each CKD stage is indicated in the legend. An ordinal logistic regression accounting for age as a covariate was fitted and confirmed a strong association between increasing CKD stage and higher protein levels, with both models showing highly significant p-values ($p < 0.001$). These results suggest that protein concentration rises in parallel with worsening kidney function, supporting its potential as a biomarker for CKD progression.

Conclusion

The detailed quantification of biomarkers provided by FibroKey™ enables better staging of CKD, supporting therapeutic decisions and drug development efforts. By offering an orthogonal confirmation of disease severity, FibroKey™ allows researchers to track disease progression with high accuracy, which is essential for assessing therapeutic efficacy and conducting clinical trials.

The FibroKey™ assay's ability to monitor fibrosis-related biomarkers in urine opens up new avenues for precision medicine in nephrology. By tracking multiple biomarkers involved in ECM remodeling and inflammation, the assay offers a non-invasive method for identifying disease progression and evaluating the effectiveness of therapeutic interventions. This approach significantly reduces the need for invasive biopsy, making FibroKey™ a key asset for both clinical research and pharmaceutical development.

Connect with Us

Partner with Inoviv to explore how FibroKey™ can support your research and drug development efforts. Contact our team to learn more about our biomarker solutions and how we can help accelerate your therapeutic strategies.

Accelerate your Drug Development Goals

Visit us at inoviv.com/FibroKey | info@inoviv.com

London: 136 High Holborn
Boston: 100 Cambridge Street
+44 (0)117 318 3961
info@inoviv.com

The FibroKey™ biomarker assay includes the following, each with specific roles in disease and fibrosis processes. All markers are validated using the InoKey workflow and provide fully quantitative results:

Biomarker	Disease and Biomarker Function
LGALS3 (Galectin-3)	Involved in fibrosis and inflammation; a marker for tissue injury and remodeling.
CD44 Antigen	Plays a role in cell adhesion, migration, and interaction with the extracellular matrix; implicated in inflammation and cancer.
Basigin (BSG) *	Regulates matrix metalloproteinases; associated with tumor progression and invasion.
SPARC	Mediates interactions between cells and the extracellular matrix; involved in wound healing and tissue remodeling.
MMP7 (Matrilysin)	Degrades components of the extracellular matrix; involved in fibrosis, cancer metastasis, and tissue remodeling.
CD9 Antigen	Involved in cell adhesion and motility; associated with cancer progression and metastasis.
CD81 Antigen *	Plays a role in cell adhesion and signal transduction; involved in immune response and cancer progression.
Osteopontin (SPP1)	Involved in inflammation, fibrosis, and cancer; regulates cell-matrix interactions.
Transthyretin (TTR)	A carrier protein for thyroxine and retinol; mutations are associated with amyloidosis.
EGFR	A cell surface receptor involved in cell growth and differentiation; implicated in cancer progression and tissue repair.
ITGB1 (Integrin beta-1)	Mediates cell adhesion to the extracellular matrix; plays a role in fibrosis, wound healing, and cancer.
FABP5	Involved in lipid transport and metabolism; linked to cancer, metabolic disorders, and inflammation.
Triosephosphate isomerase (TPI1)	An enzyme involved in glycolysis; mutations are linked to hemolytic anemia and neurological disorders.
Thioredoxin (TXN)	Reduces oxidative stress; involved in cancer, inflammation, and metabolic diseases.
Annexin A1 (ANXA1)	Mediates anti-inflammatory effects; involved in resolution of inflammation and tissue repair.
Gamma-glutamyl hydrolase (GGH)	Regulates folate metabolism; associated with cancer and metabolic disorders.
ICAM1 *	Plays a role in immune responses and inflammation; implicated in cardiovascular diseases and cancer.
Filamin-A (FLNA) *	Cross-links actin filaments; associated with tissue remodeling, cardiovascular diseases, and cancer.
Cystatin C (CYTC)	A marker for kidney function; involved in regulating protease activity in fibrosis and cardiovascular diseases.
Alpha-1-antitrypsin (A1AT)	Inhibits proteases; involved in lung diseases and liver disorders.
Interleukin-7 receptor subunit alpha (IL7RA)	Involved in immune response regulation; implicated in autoimmune diseases and cancer.
Collagen alpha-1(I) chain (CO1A1)	A major component of the extracellular matrix; involved in fibrosis and tissue remodeling.
Laminin subunit gamma-2 (LAMC2) *	A key structural component of the extracellular matrix; involved in cancer progression and tissue repair.
Matrix metallo proteinase-14 (MMP14)	Degrades extracellular matrix components; involved in fibrosis, cancer, and tissue remodeling.
MMP-2 (Matrix Metalloprotease-2) *	Breaks down extracellular matrix proteins; associated with fibrosis and cancer metastasis.
Collagen alpha-1 (III) chain (CO3A1) *	Involved in fibrosis and wound healing; a major component of scar tissue.
Cadherin-1 (CDH1) *	Mediates cell-cell adhesion; involved in cancer progression and metastasis.
Matrix metallo proteinase-9 (MMP9) *	Degrades extracellular matrix components; implicated in fibrosis, cancer metastasis, and inflammation.
Beta-2 microglobulin (B2M) *	Involved in immune response; elevated in kidney diseases and certain cancers.
Catalase (CAT) *	Breaks down hydrogen peroxide; involved in oxidative stress response and inflammation.
Fibrinogen alpha chain (FIBA)	Involved in blood clotting and wound healing; implicated in inflammation and cardiovascular diseases.
Complement C3	Part of the immune response; involved in inflammation and tissue injury.

* *Please Note: Certain markers may be below the limit of detection (LOD) in healthy human populations, with higher detectability observed in disease-specific states.*