Effect of renin-angiotensin-system blockade on urinary free light chains in patients with type 2 diabetes mellitus

Abstract : - Specific aims page

Type 2 diabetes mellitus (DM) is an epidemic. Nearly 24 million people older than 20 years of age have DM(1). DM is the leading cause of chronic kidney disease (CKD)(2). Currently, more than 20 million adults > 20 years of age have CKD (3). More than 35% of people aged > 20 years of age with DM have CKD. Measuring albuminuria remains the key tool for screening diabetic patients for nephropathy. Albumin adjusted for urinary creatinine as assessed by the spot urine microalbumin creatinine ratio (UMACR) is used to diagnose (>30 ml/min) kidney disease, assess adequacy of treatment and monitor progression of CKD. But, UMACR does not distinguish those that will have progression of CKD from those that will not. Stage of chronic kidney disease is assessed by the estimated glomerular filtration rate (eGFR). However, tight glycemic control has shown not to decrease the incidence or progression of CKD (4) and the Third NHANES data (5) reveals that not all the patients with DM with CKD have albuminuria. Limitations of UMACR have led to an international working group to call for research into new biomarkers of diabetic nephropathy(6) that either improve the likelihood of prediction of progression to CKD as a clinical marker of diabetic kidney disease. Free light chains (FLCs) are low-molecular-mass molecules (kappa and lambda light chains), which are by-products of normal immunoglobulin synthesis(7) and are normally excreted through the kidneys. Presence of light chains in the urine is a marker of tubular dysfunction. In patients with impaired kidney function, serum concentrations and urinary excretion of polyclonal FLCs have been noted to be increased. Increased excretion of FLCs and other low-molecular weight proteins [cystatin C, NGAL] in the urine may contribute to progression of chronic kidney disease. Higher Cystatin C has demonstrated to be related to development of albuminuria(8). Neutrophil gelatinase-associated lipcalin (NGAL) excretion in the urine is a marker of tubular injury in the kidney and has been shown to be elevated in subjects with type 1 and 2 DM.(9) Our data is consistent with the published data demonstrating that urinary FLC (UFLC) are elevated in patients with diabetes and hypertension as compared to healthy controls. Angiotensin-converting enzyme inhibitors (Ace Inh) and angiotensin II receptor blockers (ARB) class of drugs are renoprotective in nature and are the first line therapy for treatment of diabetic nephropathy(10). There is no longitudinal data evaluating the effect of Ace Inh and ARB class of drugs on UFLCs.

Our **hypothesis** is that UFLCs are increased in patients with DM with and without nephropathy, and that treatment with Ace Inh and/or ARB will decrease UFLCs in these patients. Additionally, we will explore the change in other low molecular weight proteins [cystatin C, and NGAL in response to treatment with Ace Inh and ARB. We propose to test the hypothesis via the following specific aims:

Specific Aims:

1- We will determine the concentration of UFLCs (kappa and lambda) and UMACR in patients with DM with eGFR > 30 min/ml not on Ace Inh or ARB treatment.
2- We will determine the concentration of UFLCs (kappa and lambda) and UMACR in patients with DM with eGFR > 30 min/ml after treatment with Ace Inh and/or ARB for three months.
3- **Exploratory aim**: We will determine the concentration of urinary cystatin C and NGAL before and after treatment in the above patients to assess their response to Ace Inh and/or ARB class of drugs.

The **short term goal** of this study is to collect preliminary data on the effect that treatment with Ace Inh and ARB class of drugs have on UFLCs in comparison to UMACR. This data will be used to apply for funding for a longitudinal study to assess the effect of continuous treatment with Ace Inh and ARB on the levels of UFLCs in comparison to UMACR. The **long term goal** is to assess and compare UFLCs as a marker of kidney damage that can be used to diagnosis kidney disease earlier than UMACR; and if UFLCs can be used for prognosis and better prediction of progression to CKD.

Project Description - **Introduction**

Diabetes mellitus and kidney disease

Diabetes mellitus (DM) is one of the leading cause of chronic kidney disease (CKD(7). It is estimated that by 2030, the annual number of patients receiving dialysis or who have had kidney transplants will exceed 2 million(11). From the 1988-1994 National Health and Nutrition Examination Survey (NHANES) data the prevalence of CKD in the US population aged > 20 years was 14.5%(12), which has increased to 16.8% according to the 1999-2004 NHANES data. Per practice recommendation, measuring albuminuria remain the key tools for screening diabetic patients for nephropathy. Albumin adjusted for urinary creatinine as assessed by the spot urine microalbumin creatinine ratio (UMACR) is used to diagnose (>30 ml/min) kidney disease, monitor progression of CKD and assess the adequacy of treatment of CKD Chronic kidney disease is defined as follows (ref): **Stage 1**: Normal GFR; GFR >90 mL/min/1.73 m2 with other evidence of chronic kidney damage; **Stage 2**: Mild impairment; GFR 60-89 mL/min/1.73 m2 with other evidence of chronic kidney damage; **Stage 3**: Moderate impairment; GFR 30-59 mL/min/1.73 m2; Stage 3 CKD should be split into two subcategories defined by (2): **Stage 4**: Severe impairment: GFR 15-29 mL/min/1.73 m2;**Stage 5**: Established renal failure (ERF): GFR < 15 mL/min/1.73 m2 or on dialysis (the term established renal failure is used instead of end-stage renal disease or end-stage renal failure).

Epidemiologic studies have demonstrated an association between poor glycemic control and kidney disease in patients with type 1 and type 2 DM (13;14). Studies have also shown that tight glycemic control slows the development of albuminuria, (15) and that improved glycemic control slows the rate of decrease in GFR. Recently, in the intensive arm of the Action to Control Cardiovascular Risk in Diabetes (**ACCORD**) trial(4), there was no reduction in the incident CKD or ESRD despite the tight glycemic control. Data from Third NHANES demonstrated that albuminuria was absent in 36% of patients with DM and CKD (5).

Thus, evidence challenges the thinking that albuminuria as measured as UMACR will always precede progression to CKD. UMACR also does not predict which patients will progress and which not to CKD. UMACR can be elevated by tobacco use, obesity, recent exercise, urinary tract infection, heart failure, uncontrolled blood glucose and hematuria**.** There is interest in research on biomarker of tubulo-interstitial injury as the pathogenic mechanisms that lead to CKD converge on a common pathway, which is tubular atrophy (16).

Urine free light chains

Free light chains (FLCs) are bi-products of normal immunoglobulin synthesis(17;18) which are predominantly removed from the circulation by the kidneys. Light and heavy chains are used to make immunoglobulins. Light chains though are produced in excess of heavy chains. The unused light chains, also known as free light chains (kappa and lambda light chain) may be present to some degree in some individuals, but should not be found in the urine of individuals with normal renal function. This is true unless there is a condition resulting in the production of excess amount of free light chains (as in myeloma) is present or if the ability of the proximal tubules to reabsorb normally excreted free light chains is decreased.

FLCs are markers of inflammation and chronic inflammation is well recognized to be associated with diabetes(17). A normal range from healthy volunteers for the level of UFLCs was established. A retrospective study conducted from patients recruited in the United Kingdom Asian Diabetes Study assessed polyclonal FLCs in patients with early diabetic kidney disease(7). Patients with T2DM had significantly raised concentrations of serum polyclonal FLCs before overt renal impairment developed ($p<0.001$). 68% of diabetic patients with normal urinary albumin: creatinine rations (ACRs) had abnormal urinary FLC: creatinine ratios. The proportion of diabetic patients with abnormal FLC: creatinine ratios rose with increasing ACR. Both urinary FLC concentrations and FLC: creatinine ratios increased significantly with microalbuminuria and proteinuria. Data by Dr. Batuman(19)on cultured cells and rodents has shown that FLCs are more toxic to kidneys than albumin. However, there is no longitudinal data evaluating the effect of drugs that are known to be renoprotective on the FLCs. Since UFLC are usually found in patients with an excess production of free light chains or in those with renal failure due to proximal tubular dysfunction, we hypothesized that free light chains would be produced in patients with diabetic nephropathy and the level of light chains in the urine may decrease with agents that are

renoprotective such as Ace Inh and ARB class of drugs. Ace Inh and ARBs are the first line therapy for treatment of kidney disease. There are limitations of UMACR that have led to an international working group to call for research into new biomarkers of diabetic nephropathy(6). To assess the utility of UFLCs as a marker of diagnosis and prognosis of CKD, we propose the following specific aims:

Specific Aims:

1- We will determine the concentration of UFLC (kappa and lambda) and UMACR in patients with DM with eGFR > 30 min/ml not on Ace Inh or ARB treatment.
2- We will determine the concentration of UFLCs (kappa and lambda) and UMACR in patients with DM with eGFR > 30 min/ml after treatment with Ace Inh and/or ARB for three months.
3- **Exploratory aim**: We will do exploratory analysis on the levels of cystatin C and NGAL to assess the change in their levels after treatment with Ace Inh and/or ARBs and correlate it to the change in UMACR and UFLCs in the above patients.

Innovation:

Whether albuminuria (as measured by the UMACR) is a good surrogate marker for progression of CKD in DM remains in question. There is a need for biomarkers that can detect kidney disease in earlier stages than UMACR and can reliably predict the progression of CKD. Analysis of biomarkers specific of tubulointerstitial kidney injury, the common pathway of progressive kidney disease will give us insight into the potential of such a surrogate marker of CKD. The presence of free light chains in the urine samples of patients with diabetes may be an indication of diabetic kidney disease if UMACR < 30. The effect of renoprotective drugs on UFLCs warrants to be documented to follow these patients with CKD and thus explore the potential of UFLC as a marker of kidney disease.

Supporting data

We hypothesized that urinary polyclonal free light chain excretion is elevated in early diabetic kidney as compared to controls and that urinary polyclonal free light chains excretion is elevated in early diabetic nephropathy before GFR declines. The objective was to investigate the excretion of urinary free light chains (UFLC) in men and women, with and without DM, with a body mass index ≤27 or ≥30. It was a cross-sectional study, with males and females between the ages of 18 and 70. Urine κ and λ FLC concentrations were measured by nephelotmetry, on a Dade-Behring BN ™ II Analyzer using a particle-enhanced, high-specificity, homogenous immunoassay. In our study(20), there were three groups; controls (group I), obese without DM (group II), and obese with DM and HTN (group III). In group III, the mean HbA1c was 7.4 ± 1.3%. See table 1 for the results.

Table 1: Results of the UFLCs and UMACR

	I–Healthy Controls	II–Obese without DM	III- Obese with DM and HTN
N	10	40	30
Females	5	20	15
Age (years)	37.0 ± 10.5	45.7 ± 11.6	57.3 ± 7.0
BMI	22.5 ± 2.8	38.2 ± 7.5	41.6 ± 6.9
S. Creatinine (mg/dL)	0.79 ± 0.2	0.9 ± 0.3	0.9 ± 0.9
UMACR (mg/g)	9.2 ± 7.54	23.7 ± 61.6	27.2 ± 70.6
Urine Kappa (mg/L) † *	14.7 ± 11.1	34.0 ± 63.7	51.0 ± 71.8
Urine Kappa/Cr (mg/L) † *	0.01 ± 0.01	0.03 ± 0.06	0.04 ± 0.03
Urine Lambda (mg/L) †	5.8 ± 14.0	2.3 ± 3.9	8.7 ± 16.5
Urine Lambda/Cr (mg/L)	0.002 ± 0.003	0.002± 0.003	0.006 ± 0.013
Urine Kappa + Lambda (mg/L) †	20.4 ± 15.1	36.3 ± 66.9	59.7 ± 76.9
Urine Kappa + Lambda/Cr (mg/L) †	0.01 ± 0.01	0.02 ± 0.07	0.04 ± 0.04

UMACR= urine microalbumin creatinine ratio

† = $P < 0.05$ between groups I and III

* = Trend toward significance between groups I and II [(free Kappa, $P = 0.07$), Kappa/Cr, $P = 0.08$)]

Results suggest excretion of urinary polyclonal free light chains is increased in obese subjects with DM and hypertension as compared to normal controls.

Limitations of this data include the cross-sectional design and use of Ace Inh and/or ARB class of drugs by 75% of the subjects in groups II and III. Therefore, the effect of Ace Inh and/or ARBs on the mean change of UFLC as compared to UMACR could not be assessed.

Approach

The proposed research project is aimed at studying the effect of anti-hypertensive medications on UFLCs as compared to UMACR. We will recruit subjects with CKD stages I, II, and III. This will allow us to have a direct comparison between the two variables (UFLCs and UMACR) across the above three stages of CKD, excluding stages IV and V. The reason for excluding patients with stages IV and IV is that decreased clearance of the UFLCs will confound the results.

Inclusion Criteria:

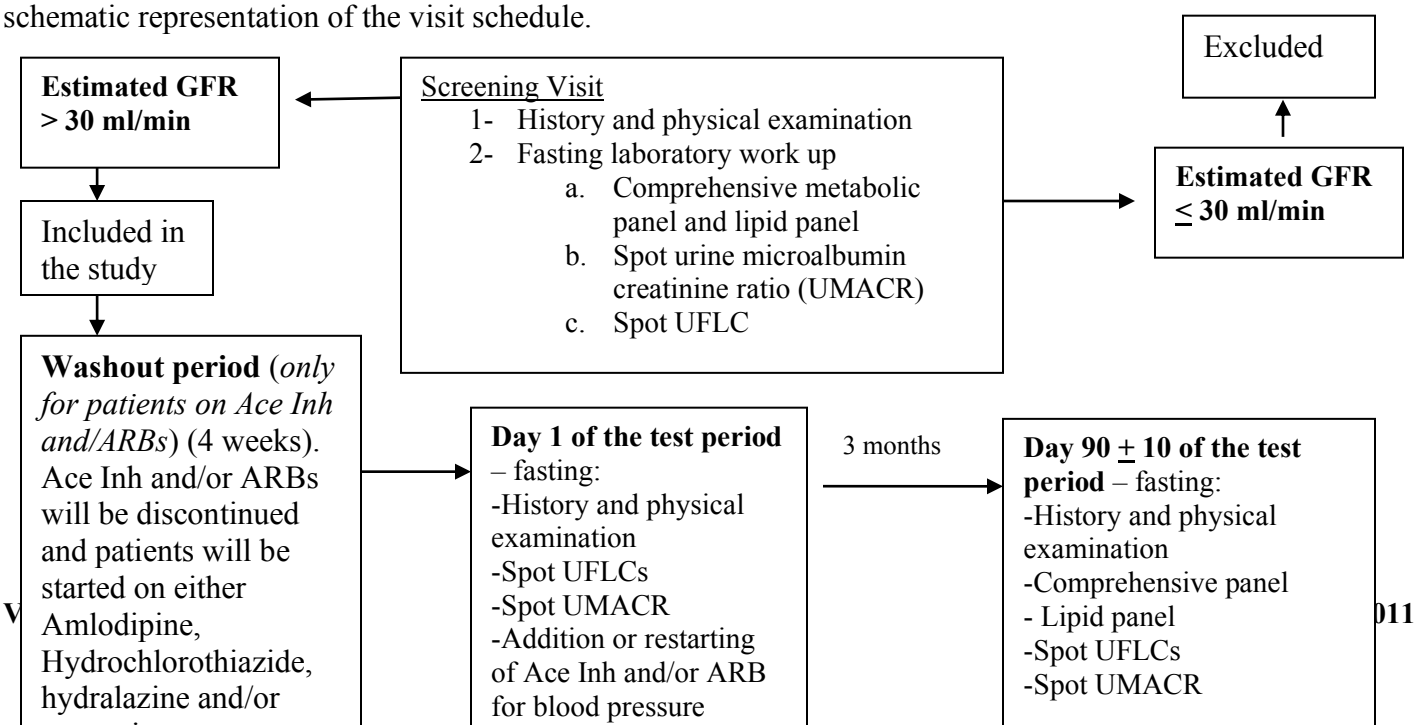
1- Men and women with DM, type 1 or type 2.
2- Age 18- 75
3- Estimated glomerular filtration rate (eGFR) > 30 ml/min
4- Hypertension
5- Use of Ace Inh and ARB for control of blood pressure who are willing to be placed on alternate drug(s) in the washout period for blood pressure control.

Exclusion Criteria:

1- Pregnancy
2- Patients with chronic kidney disease stage with eGFR ≤ 30 ml/min (CKD stage IV and V)
3- Nephrotic range proteinuria (urinary protein > 3.5 gm/day)
4- History or renal transplantation
5- History of multiple myeloma
6- Known history of hypersensitivity reaction or intolerability to Ace Inh or ARB, Amlodipine, Terazosin, Hydrochlorothiazide, and/or Hydralazine.

Research Design

This is a prospective, interventional study involving patients with either type I or type 2 DM and hypertension, which will be recruited from the outpatient clinics and the Tulane volunteer registry. Patients who meet the inclusion criteria on evaluation of blood work in the last 90 days of the visit will then undergo screening. Information from the most recent laboratory data will be collected. There will be no genetic testing. The study is designed to include patients with and without diabetic nephropathy. All pertinent medications will be recorded as this information will be very important in interpreting the results of the data analyses. Below is a schematic representation of the visit schedule.



Washout period
In the washout period, if Ace Inh or ARB classes of drug are part of patients' antihypertensive regimen, then the Ace Inh and/or ARB medication(s) will be discontinued. The patient will be started on alternative therapy (Amlodipine, Hydralazine, Terazosin and/or Hydrochlorothiazide) for control of their blood pressure. **The goal for their blood pressure will be set at < 140/90 mm/Hg**.

Test Period
On day 1 of the test period, patients will be advised not to take their blood pressure medication(s) prior to the study visit. During the study visit, urine sample will be collected for analysis of spot UFLCs and spot UMACR. Additional 40 ml of urine and 10 ml of blood will be collected and stored for further analysis. The stored urine will be analyzed for cystatin C, NGAL. Urine and blood will be analyzed for other markers of obesity, diabetes, cardiovascular and kidney disease depending on the availability of funding.
After the blood and urine sample have been collected in a fasting state, those patients that were on Ace Inh or ARB prior to the washout period, will be started on the same regimen (Ace Inh and/or ARB). The alternate medication(s) that were started during the washout period will be discontinued.
Amlodipine, Hydrochlorothiazide, Hydralazine, Terazosin, Lisinopril and Losartan are all available in generic formulations. The dose of the drug and the decision to titrate the medication(s) for appropriate control and/or addition of medications will be decided by the principal investigator.

Methodology
Urine samples collected from patients will be aliquotted and stored in a -70oC deep freezer until the assays are performed. The following assays will be used to measure the respective markers.

1- A new latex-enhanced immunonephelometric assay measuring free k and l light chains as a free form (Freelite®, The Binding Site, San Diego, CA) has been developed. We have measure urinary polyclonal k and l FLC of immunoglobulins by the nephelometric immunoassay.
2- Microalbuminuria[Micro-albumin (MAB) (Orgentec Diagnostic, Mainz, Germany], and urinary excretion of low molecular weight proteins, cystatin C (CSC) (Arbor Assays, Ann Arbor, MI) and NGAL (R&D Systems, Minneapolis, MN) will be quantified by competitive solid phase enzyme immunoassay (ELISA) in accordance with the manufacturer's instructions.
3- Urinary creatinine (CRN) levels will be measured by Jaffe's method.
4- The urine free light chains: creatinine ratio as well as the urine microalbumin: creatinine ratio will be calculated and analyzed.

Analysis
Power and sample size
We plan to recruit 150 patients. Assumptions are: power = 0.8; alpha = 0.05, with 30% reduction in mean response being taken as significant. There is no data on the correlation of pre and post measures. We have set the correlation to 0.7 as Hutchison et al have demonstrated the correlation between urinary kappa and lambda light chains to be 0.88 with UMACR(7). The standard deviation from our data were ± 72 mg/L for urinary kappa light chains and ± 17 mg/L for urinary lambda chains in patients with DM and HTN. Assuming similar standard deviations in our sample for the proposed study, the sample size of 150 will allow us 80% power to detect a correlation coefficient of 0.7 between two normally distributed numerical variables when type I error is 5%.
Statistical analysis

Summary statistics such as mean, standard deviations, ranges, and percentiles of numerical variables will be given. Plots and goodness-of-fit tests will be generated to examine the distributions of the variables. Data will be log-transformed to normalized variables when necessary. If transformations do not resolve serious distribution problems, then nonparametric techniques will be considered as alternatives to parametric techniques. Outliers and influential points will be identified and examined.
Prevalence of incidence of interests will also be provided. Bar-plots and pie-charts will be produced for visual illustrations. Two-sample t-tests will be used to detect differences in numerical outcomes between groups, and chi-square tests will be employed to compare the frequencies of categorical outcomes. Linear or logistic regression models will be fitted to examine effects of covariates. Stepwise model selection methods will be used to identify for potential risk factors or prognostic variables. Residual analysis will be followed to validate the assumptions. All analyses will be performed using SAS version 9.1. Pearson or Spearman's correlation coefficients will be estimated between cystatin C and NGAL and traditional markers (blood pressure, lipids, UMACR, etc.) and UFLCs.
This data will be presented in the form of poster(s) at scientific meeting(s) and used for peer reviewed publications and future grant applications.

Subject recruitment and safety
Patients will be recruited from the outpatient clinics and the Tulane volunteer registry. All patients will sign the informed consent prior to starting the study. **Data safety monitoring board** will be formed that will review the data every six months to ensure patient safety and recommend continuation or discontinuation in the study. All patient information and data will be stored in a safe place under lock and key.
The target of blood pressure for patients during the washout period of the study is < 140/90 mm/Hg as per treatment guidelines(10). It is important to change the medications, as Ace Inh and ARB are the first line therapy in patients with DM. Since the focus of the proposed project is to study the effect of Ace Inh and ARB on UFLC, collection of UFLC with Ace Inh and/or ARB will confound the results.
To address this, patients will be advised to check their blood pressure at least 3 times / week either at home or in a facility such as a pharmacy. Given the variability in the machines, they will be advised to be consistent about where they check their blood pressure. If the blood pressure is > 130/80 mm/Hg, they will be advised to contact the principal investigator. The principal investigator will then make appropriate changes to their medication regimen.

Significance
Inclusion of patients with many different clinical (DM and HTN, with and without nephropathy) and biochemical variables will allow us to evaluate the prevalence of these clinical and biochemical parameters, including albuminuria and relate it to the UFLCs depending on the renal function. In those patients in whom UMACR < 30 upon entering the study, it will allow us to collect data on the effect of anti-hypertensive medications on UFLCs before kidney damage starts as compared to UMACR. This cohort of patients can be followed over time to assess the utility of UFLCs as a marker of kidney damage in comparison to UMACR.
In those subjects, that do have established kidney damage (diagnosed by UMACR > 30); data collected at the end of the study will allow us to study the effect of anti-hypertensive medications on UFLCs as compared to UMACR. The next step for this cohort of patients would be to follow them longitudinally to examine the role of UFLCs as a prognostic marker of progression of kidney damage in comparison to UMACR. **Data from the proposed study will be used to apply for funding for a longitudinal study.**

Key Personnel
Drs. Batuman and Li (Co-Investigators) – have expertise in UFLCs, kidney disease and have the necessary laboratory resources.
Dr. Vivian Fonseca (Consultant) – has expertise in DM, insulin resistance, obesity and conducting clinical trials.

Biostatistician (TBA) – we will consult with a biostatistician for data analyses and proper interpretation. The biostatistician will also help us use the obtained data for further grant application preparation.

Description of the relevance of the project to long-range goals

Public Health Relevance

There are now more than 385,000 people in the U.S. with ESRD and the burden of ESRD is growing. Treating ESRD imposes a large economic burden on patients, the health care system, and society. Diabetes mellitus is the leading cause of ESRD. There is evidence that earlier stages of CKD can be detected and treated and that adverse outcomes of CKD can be prevented or delayed(21) . Data on urinary free light chains, which are a marker of tubular dysfunction, is sparse with no data on the effect of treatment with Ace Inh and ARB on UFLC. Obtaining such data as proposed in this translational research project is critical as Ace Inh and ARB class of drugs are the standard of care in the treatment of patients with DM and kidney disease. Data obtained from this study will be used to apply for peer-reviewed grant applications for funding for a larger, longitudinal study to study if UFLC can be used as a prognostic marker in monitoring the progression and adequacy of treatment of kidney disease in comparison to urine microalbumin creatinine ratio. The long range goal is to study the utility of UFLC as an early biomarker of kidney disease.

Relevance for Tulane investigators

Dr. Thethi has been able to recruit 590 patients for her current study investigating obesity and biomarkers of kidney damage. Data from this cohort of patients has yielded preliminary results on several biomarkers and given insight into some disease processes linked to obesity associated kidney disease. Drs. Batuman and Li have expertise in urinary free light chains; and have laboratory equipment and resources available for running the assays for urinary free light chains. This study utilizes the expertise of investigators at Tulane with the common interest in finding biochemical monitoring modalities to detect kidney disease in the earlier stage and to find modalities to better monitor its progression.

In establishing this **collaboration** for pilot funding with Dr. Batuman and obtaining preliminary data through the proposed **translational research project following the model of interdisciplinary research**, we will be able to apply for a longitudinal study investigating the role of UFLC as a biomarker of kidney damage and prognosis.

Budget Request and Justification

Personnel		% Effort	Salary	Fringe	Total
Medical Research Technician		15%	$ 3,675.00	$ 827.00	$ 4,502.00
Data Manager		2%	$ 758.00	$ 171.00	$ 929.00
Research Coordinator		1%	$ 585.00	$ 132.00	$ 717.00
Supplies/Materials					$ 39,885.00
Travel					$ 1,317.00
Publication Costs					$ 150.00
IRB Fees					$ 2,500.00
Total					$ 50,000.00

Personnel

Tina K. Thethi, MD, MPH: Principal Investigator-is not requesting salary support.
Co-Investigators (Vecihi Batuman, MD, and Min Li, MD, PhD), Consultant (Vivian Fonseca, MD) and Biostatistician (TBA): are not requesting salary support.
Robin Bye, RN-Research Coordinator: 1% effort. Once patients are referred for the study, the coordinator will be responsible for following up with the patients, randomization, and scheduling study protocol related visits. The coordinator will be in close communication with the principal investigator of the study.
Jennifer Huber-Data Manager: 2% effort. Will be responsible for obtaining and compiling the data in an accessible, but secure manner and location.
TBA-Medical Research Technician: 15% effort. Will be responsible for running urinary free light chain assays.

Supplies and Materials

Patient Visits: Patients will be given a $25 reimbursement for travel expenses associated with each visit. Patients are expected to have three visits in the course of the study which results in a total reimbursement of $75 per patient. The study will include 150 patients; therefore, the total cost for patient reimbursements will be $11,250.00.
Screening and Laboratory Tests: Each patient will receive three CMP tests at a cost of $4.50 each for a total of $2,025.00. Each patient will receive two lipid panels at the cost of $10 each for a total of $3,000.00. Approximately 100 patients will receive a B-Hcg at a cost of $13.35 each for a total of $1,335.00. Each kit for UFLC will test 30 patients, and each patient will receive one test; therefore, 5 kits will be needed at a cost of $1,800.00 each for a total of $9,000.00. Each kit for urine creatinine will test 30 patients, and each patient will receive one test; therefore, 5 kits will be needed at a cost of $180.00 each for a total of $900.00. Each kit for urine microalbumin will test 30 patients, and each patient will receive one test; therefore, 5 kits will be needed at a cost of $475.00 each for a total of $2,375.00. $8,000 has been set aside for miscellaneous variables in exploratory analyses.
Medicine: Patients currently taking Ace Inh or ARBs as part of their daily regimen prior to the washout period will need to be taken off these drugs. The study is meant to study the effects of these drugs, so the patients' systems must be clear of them when the study begins. These patients will be placed on other drugs (hydralazine or terazosin) during the washout period.

Travel: Allotted funds will be used to travel to scientific meetings to present the data.

Anticipated timeline and completion

Months	1-6	7-12	13-18
Subject recruitment	X	X	X
Obtaining and analyzing pilot data		X	X
Presenting pilot data at scientific meetings		X	X
Peer-reviewed publications		X	X
Preparation of peer reviewed grant application(s) for a larger longitudinal study			X

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