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Urinary metabolomics: to distinguish diabetic kidney disease from non-diabetic kidney disease

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Background and aims: Differential diagnosis of diabetic kidney disease (DKD) and non-diabetic kidney disease is essential for appropriate therapeutic intervention. However, currently, the biopsy is the only reliable method, which is advisable to only a subset of the patient population and often fraught with risk. Urinary metabolic biomarkers can provide a non-invasive alternative that is highly warranted. So far no study has ever reported a comparison of urinary metabolic signatures of biopsy-confirmed DKD and NDKD subjects. Aim: Comparison urinary metabolic signatures of biopsy-confirmed DKD and NDKD subjects and diabetic controls with normal kidney function to identify putative biomarkers for differential diagnosis of DKD and NDKD.

Materials and methods: Urine samples from 17 biopsy-confirmed DKD, 4 NDKD and 7 age, body-weight and BMI-matched diabetic subjects with normal kidney function samples were deproteinated, derivatized and analyzed in random order by GCMS along with intermittent injection of quality control samples. Features were extracted and analyzed using nonparametric Kruskal-Walis test with Dunn's correction for multiple testing

Results: Creatinine-normalized abundance of two urinary metabolites (M1, M2 and M3) were found to be depleted in DKD ($P < 0.004$, <0.006, and < 0.01, respectively) as well as NDKD ($P<0.02$, <0.04, and <0.01, respectively) with respect to diabetic control subjects. On the other hand, M4 was elevated ($P< 0.03$) and M5 was depleted ($P < 0.02$) exclusively in DKD subjects while M6 ($P<0.01$) and M7($P<0.03$) were exclusively depleted in NDKD subjects with respect to diabetic controls. The level of M6 in NDKD subjects were also significantly lower ($P<0.03$) compared to DKD subjects. In addition, another three metabolite M8 ($P<0.05$), M9 (0.03) and M10 (0.02) were found to be exclusively depleted in NDKD samples compared to DKD subjects only. Analysis of fragmentation pattern indicated that these ten metabolites putatively belong to central carbon metabolism, fatty acid metabolism, purine metabolism in addition to metabolites exclusively of non-endogenous (microbial) origin.

Conclusion: Our pilot study suggests that urinary metabolomics analysis may help to distinguish DKD and NDKD subjects from diabetic controls with normal kidney function. Our results warrant validation in a bigger cohort (ongoing).

Supported by: RSSDI

Disclosure: **S. Ghosh:** None.

Institute of Post Graduate Medical Education & Research
244, A.J.C. Bose Road, Kolkata – 700020.
IPGME&R Research Oversight Committee
(Institutional Ethics Committee)

Memo No. IPGME&R/IEC/2017/403

Date: 21.08.2017

Dr. Sujoy Ghosh
Associate Professor
Department of Endocrinology
IPGME&R, Kolkata – 700020

Dear Dr. Ghosh,

A meeting of the Institutional Ethics Committee of IPGME&R, Kolkata, was held on 12.08.2017 at 12:00 Noon in the IPGME&R Dean's Office. In this meeting the members considered the protocol of your project titled:

Urine metabolomics and proteomics for differentiation of diabetic from non-diabetic kidney disease (biopsy proven) in patients with type 2 diabetes with chronic kidney disease.

The following additional documents were reviewed:

- Informed consent document and form in English.
- Informed consent document and form in Hindi.
- Informed consent document and form in Bengali.

After deliberations and review the committee took the following decision regarding your project:

Approved

The Committee understands that this is to be an academic research project without any commercial sponsor.

It is placed on record that the decision regarding your proposal was unanimous and therefore did not require any voting procedure. Members absent have reviewed the same documents and have not sent any note of dissent or objection regarding your proposal.

Additional points, if any, mentioned on Page 2 are to be noted.

Continued on Page 2

Continued from Page 1

Additional points to be noted

- The Committee expects that any amendments to the Study Protocol, Informed Consent documents or other relevant documents would be brought to its notice.
- A brief project completion report is to be submitted to the IPGME&R Research Oversight Committee. If project duration exceeds 1 year from commencement, a brief annual progress report should also be submitted.
- IPGME&R Research Oversight Committee is registered with Central Drugs Standard Control Organization (CDSCO), Government of India, in consonance with Rule 122D of the revised Drugs & Cosmetics Rules 1945 – Registration No. ECR/35/Inst/WB/2013/RR-16. It functions in accordance with revised Schedule Y and Indian Council of Medical research (ICMR) guidelines.

List of institutional ethics committee members who attended the meeting on 12.08.2017

SN	Name & role in the committee	Gender	Designation
1	**Dr. Hemanta Kumar Majumder** [Scientist & Chairperson]	Male	Senior Scientist, Indian Institute of Chemical Biology, Kolkata
2	**Prof. Amal Kanti Das** [Pharmacologist]	Male	Dean of Student Affairs and Head, Department of Pharmacology, IPGME&R
3	**Prof. Biswanath Kahali** [Forensic Expert]	Male	Head, Department of Forensic Medicine, IPGME&R, Kolkata
4	**Prof. Swati Chakravarti** [Clinician – Pediatrician]	Female	Consultant, Institute of Child Health, Kolkata
5	**Prof. Jayanta Chatterjee** [Clinician]	Male	Head, Department of Nuclear Medicine, IPGME&R
6	**Prof. Amal Kumar Santra** [Basic medical scientist]	Male	Scientist, Department of Gastroenterology, IPGME&R
7	**Prof. Rathindranath Dutta** [Clinician]	Male	Consultant Dermatologist and Former Head, Department of Dermatology, IPGME&R
8	**Dr. Bobby Pal** [Public health expert]	Female	Assistant Professor, Department of Preventive & Social Medicine, All India Institute of Hygiene & Public Health, Kolkata
9	**Mr. Debdut Mukherjee** [Legal expert]	Male	Advocate, Calcutta High Court
10	**Mr. Arunangshu Shekhar Jana** [Social worker]	Male	Social worker, Mahendraganj, Dist. South 24 Parganas
11	**Dr. Nila Majumdar **** [Lay person]	Female	Bengali teacher, Kolkata
12	**Prof. Avijit Hazra** [Pharmacologist & Member secretary]	Male	Professor, Department of Pharmacology, IPGME&R

** Included in the Committee in place of Mr. Swapan Kumar Sarkar from 16.12.2016.

Avijit Hazra 21/08/2017

Dr. Avijit Hazra – Member Secretary
IPGME&R Research Oversight Committee

Member Secretary
Institutional Ethics Committee
Institute of Postgraduate Medical
Education & Research (IPGME&R)
Kolkata-700020

RSSDI

Research Society for the Study of Diabetes in India

RSSDI/HQ/Grants/2018/510 Date- 19.04.2018

To,
Dr.Sujoy Ghosh,
Associate Professor,
Dept.of Endocrinology and Metabolism,
4th Floor, Ronald Ross Building,
IPGMER & SSKM Hospital, Kolkata-20.
Mobile No – 9674625823

Sub:- Disbursement letter for the Research project entitled "Identification of non-invasive signatures for differential diagnosis of biopsyproven diabetic nephropathy and non-diabetic kidney disease by metabolomics and peptidomics approach" with a project duration of 2 year.

Dear Dr.Sujoy Ghosh,

Please find enclosed cheque no"834426" dated 19/04/2018 in favour of **"STUDY OF DIABETIC NEPHROPATHY"** - for sum of **Rs. 747500 /- (Seven Lakh Fourty Seven Thousand Five Hundred Only)** drawn on Punjab National Bank for your project entitled "Identification of non-invasive signatures for differential diagnosis of biopsyproven diabetic nephropathy and non-diabetic kidney disease by metabolomics and peptidomics approach" as 1st year Installment of 6 months.

You are requested to submit the complete report of your work along with the Utilization Certificate and Statement of Expenditure at the end of 1st year of the project.

Kindly acknowledge receipt of the same.

With Regards,

Yours Sincerely,

Dr .Brij Mohan Makkar,
Secretary, RSSDI

RSSDI Secretariat :
Dr. Makkar's Diabetes & Obesity Centre
A-5B/122, Paschim Vihar, New Delhi-110063 (India)
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PROPOSED TITLE

Identification of non-invasive signatures for differential diagnosis of biopsy-proven diabetic nephropathy and non-diabetic kidney disease by metabolomics and peptidomics approach

INVESTIGATORS

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Introduction

Chronic Kidney disease (CKD) in diabetes is one of the most prevalent microvascular complications (1) which lead to end-stage renal disease (ESRD) worldwide (2). Among all cases of kidney failure, about 44% is associated to diabetes. Diabetic patients with kidney disease may suffer not only from kidney failure but also cardiovascular disease associated morbidity and mortality and high treatment cost (3). Kidney disease in diabetes is the most common complication among the both type of diabetes, which usually manifest after 10-15years after diagnosis to Type-1 diabetes or Type-2 diabetes (4).

Differential diagnosis of diabetic vis-a-vis non-diabetic kidney disease based on clinical manifestation and biochemistry is often challenging. This is believed to lead to underrepresentation of the non-diabetic kidney disease in epidemiological studies. More importantly, misclassification might lead to deleterious consequences for the patient as the management regimes of these two diseases are different altogether. Since histopathological investigation, the gold standard for diagnosis, is invasive with high risk and the facility for biopsy is not available commonly, identification of a non-invasive or, at least, minimally-invasive signature may significantly improve therapeutic outcome. It may also yield tools for disease surveillance and therapeutic monitoring.

1. Ralph A. DeFronzo, El Ferrannini, Paul Zimmet, K. George M. M. Alberti, International Textbook
of Diabetes Mellitus, Fourth Edition, 2015, DOI: 10.1002/9781118387658
2. Prakash J et al., J Assoc Physicians India. 2013 Mar; 61(3):194-9.
3. Nassirpour R t al., Food ChemToxicol. 2016 Dec; 98(Pt A):73-88.
4. Das U et al., Indian J Nephrol 2012; 22:358-62

Review of existing literature

Metabolomics

Previous studies have revealed changes in metabolic signature associated with kidney damage (reviewed in 1, 2). Most of these studies dealt with acute kidney injury (3, 4), chronic kidney disease (5, 6) or renal carcinoma (7, 8). Based on serum and urinary metabolomic analysis, results of earlier studies (1, 9) also suggested dysregulation of a number of metabolic pathways including glycolysis, TCA cycle, purine metabolism, lipid metabolism and amino acid metabolism to be associated with diabetic kidney disease. Using metabolomics, Sharma et al showed evidence for mitochondrial dysfunction, which would contribute to cell death and, thus, impairment of kidney function (10). Putative early stage non-invasive biomarkers for diabetic kidney disease have also been identified through urinary metabolomics (11). However, **no such study has been reported for non-diabetic kidney disease in patients with T2DM. Surprisingly, the patient recruitment criteria of most of these aforementioned studies were not based on biopsy confirmation of diabetic kidney disease. Evidently, there has also been no comparative analysis of metabolomic signature associated with biopsy-confirmed diabetic and non-diabetic kidney disease**.

Pathogenesis of diabetes is associated with distinct sets of dysregulation of metabolic machinery involving multiple organs primarily involving liver, pancreas, muscle and adipose tissue. Diabetic kidney disease is a result of this systemic derangement at a later stage. Since kidney plays a major role in filtration and re-absorption of small molecules, impairment of kidney function is expected to affect the metabolic signature in urine as well as serum. The absolute and relative abundance of different metabolites in biofluids will depend not only on kidney function but also on function of other organs involved in their homeostasis. Thus, in case of diabetic kidney disease, change in urinary metabolic signature is expected to have contribution from impaired kidney function as well as aberration in metabolic machinery of other organs such as liver, pancreas, muscle and adipose tissue. On the other hand, non-diabetic kidney disease may not be a result or lead to extensive dysregulation of metabolic machinery in aforementioned organs. Thus, the urinary metabolomic signaturein non-diabetic kidney disease may be distinct from that associated with diabetic kidney disease.

Peptidomics It has long being postulated that uremia is associated with change in accumulation and excretion of 'middle molecules' (12). Although earlier the molecular weight range of these molecules was suggested to be 500-2000 Da, recently it has been revised to be 500-60000 Da (13). A number of studies have, in fact, looked into changes in urinary proteome to find biomarkers for chronic kidney diseases including diabetic nephropathy (14-16). However, it should be noted that, abnormal protein excretion in kidney disease is typically expected to occur after significant glomerular damage has taken place, i.e., in advanced stages. On the other hand, peptides are filtered from blood even at normal physiological condition. Therefore, any changes in circulating or kidney-derived peptides are likely to appear in urine early during pathogenesis of chronic kidney disease and act as early biomarkers. Peptides, being degradation products of protein, are also expected to provide more robust signature compared to proteins themselves, which may undergo further modification and/or degradation during storage in bladder. In addition, emerging evidence also suggest an important role of ubiquitin-mediated proteosomal degradation in renal dysfunction (17-18). Put together, these suggest a change in proteolytic products, i.e., peptides, associated with renal dysfunction. In fact, an earlier study analyzed human urine samples to identify peptides that may serve as putative biomarkers for chronic kidney disease (19). Two recent studies have also revealed changes in urine peptidome in rodent models of diabetic nephropathy (20-21). Given the distinct nature distinct nature of kidney damage in DKD and NDKD, the resulting peptidomic signature may be different between these two diseases. However, till date **there has been no report of comparative analysis between human urinary peptidome associated with biopsy-confirmed DKD and NDKD**. This study proposes to carry out global metabolomic and peptidomic profiling to identify signatures that could be exploited for differential diagnosis of diabetic and non-diabetic kidney disease.

Based on the brief background and literatures mentioned above, we hypothesize that
"**Diabetic kidney disease (DKD) and non-diabetic kidney disease (NDKD) bears *distinct* metabolomic and peptidomic signatures**".

To test the hypothesis, we specifically aim

(i) To characterize the difference in urinary metabolome of biopsy-confirmed diabetic and non-diabetic kidney disease patients.

We would analyze the difference in metabolic signature associated with DKD and NDKD using urine samples.

(ii) To characterize urine peptidome of biopsy-confirmed diabetic, non-diabetic kidney disease patients.

We would like to examine the change in excretion of the low and middle molecular weight peptides in DKD vis-a-vis NDKD. Their correlation with pathology biochemical parameter and prognosis will be examined.

(iii) To analyze the robustness of differential metabolomic and peptidomic signatures with respect to sample collection, storage and handling.

We would like to assess the effect of sample collection timing, protocol, storage temperature and duration etc on the ability of the metabolic and peptidomic signatures in differentiating DKD and NDKD cases.

Significance

Since NDKD often has a much better prognosis compared to DKD and may be curable, identification of robust noninvasive biomarkers that could help in differential diagnosis of DKD and NDKD in diabetic patients will have an immediate impact on patient stratification and choice of treatment. This could not only increase the speed of initial diagnosis, but also provide a way for monitoring therapeutic outcome. Eventually, potential use of noninvasive biomarkers may aid in population-level screening and surveillance.

National and International Status: As described above, there has been no report at either national or international level on identification of metabolomic and peptidomic biomarkers for differential diagnosis of DKD and NDKD in diabetic patients using biopsy-confirmed cases. To the best of our knowledge, there is no such study ongoing at the national level.

Methodology

Study design

Cross-sectional, Hospital based.

Study settings

OPD of Department of Endocrinology and Metabolism and Department of Nephrology, IPGME&R and SSKM hospital

Study population

Patients who are attending OPD of Department of Endocrinology and Metabolism and Department of Nephrology, IPGME&R and SSKM hospital. We shall recruit 50 biopsy confirmed DKD and 50 NDKD patients

Sampling methods

Among considered study population, who gave consent would be screened by clinical standard criteria of DKD and NDKD as described (1) and grouped as true DKD and true NDKD (confirmed by biopsy).

Sampling techniques Purposive sampling

Inclusion criteria

Age >18 years, both male and female type2 DM patients with proteinuria and /or renal dysfunction (30-90 ml/min or 1.73m^2) GFR

Exclusion criteria

Type-1 diabetic patients, patient unwilling for kidney biopsy and sampling of studies and end stage renal disease (GFR <15ml/min/1.73ml for more than 3 months)

Reference

1. Thijs W et.al, JASN (Journal of American Society of Nephrology) April 1, 2010; 21, 4556-563

Study Techniques

After obtaining the certificate of clearance from ethical committee study population is selected randomly among the patients who are attending OPD and diabetic clinic of SSKM hospital, West Bengal. Morning first-pass urine samples will be collected. Three 1 ml aliquot of the sample will be flash-frozen immediately upon arrival and stored at -80°C until further analysis. 3.6 ml urine will be centrifuged (1.8 ml each in two micro-centrifuge tubes) and three aliquots of 1 ml supernatant will be flash-frozen and stored at -80°C. On basis of reported biopsy confirmation, samples will be grouped into DKD and NDKD. All the data of routine biochemical tests for diabetes profile and CKD profile will be kept for future use. The food and xenobiotic exposure data (particularly, drugs) of the patient the preceding day and on the day of the sample collection will be recorded. Metabolomic and peptidomic analyses will be carried out in collaboration with Dr. Soumen Kanti Manna, Saha Institute of Nuclear Physics, Kolkata as described below.

Kidney biopsy and assessment of biopsied tissue

Biopsy, after written consent will be done according to standard method. After appropriate sectioning and staining, slides will be evaluated by expert pathologist following the criteria described by Thijs et al.

References

1. Thijs W et.al, JASN (Journal of American Society of Nephrology) April 1, 2010; 21, 4556-563

Metabolomics of urine samples

Global metabolic profiling of bio-fluids is a very challenging task owing to the wide range of chemical diversity, abundance and stability of constituent molecules. There is not a single platform that can capture it entirely. Gas chromatography-coupled with mass spectrometry has been a seasoned technique in analysis of small molecules. The availability of fragmentation library helps a lot in establishing putative identity of a molecule even without authentic standard. In addition, sensitivity of GC makes it suitable for trace level analysis. However, GC-MS cannot be used for molecules that are not volatile. Besides, it is also quite time-consuming and, therefore, relatively low-throughput. These issues could be substantially addressed by the use of tandem liquid chormatography mass spectrometry platforms. Particularly, recent advances in ultra-performance liquid chromatography (UPLC) have dramatically improved throughput as well as the sensitivity of analysis. It can be coupled with electrospray ionization time-of-flight mass spectrometry (ESI-TOF-MS) to achieve highly accurate mass measurement that helps towards identification of the analyte. In addition, use of different types of columns with varying affinity for molecules of different polarity along use of both positive and negative ionization modes, further expands the coverage of metabolomic space. On the other hand, the innovation of fast-scanning triple-quadrupole mass spectrometers has enabled simultaneous monitoring of multiple characteristic fragmentation reactions to quantitate numerous metabolites at the same time. This project will employ our expertise (21-23) in the use these state-of-art technologies to identify and quantitate putative biomarkers that could distinguish between diabetic nephropathy and non-diabetic kidney damage in diabetic patients.

Urine samples will be deproteinated using acetonitrile and analyzed using UPLC-ESI-MS. For GC-MS analysis, urine samples will be derivatized with MOX and silylating reagents (BSTFA or MSTFA containing TMCS) following Urease treatment.

Chromatographic and mass spectrometric data will be aligned, deconvoluted and binned to extract features, which will be subjected to multivariate analysis. SIMCA-P+ (Umetrix, Umea, Sweden) and Random Forest will be used for multivariate statistical analysis of metabolomic data matrix. Unsupervised (PCA) as well as supervised (PLS-DA and OPLS-DA) pattern recognition analysis will be performed to identify signatures that could distinguish pathologies. The chemical identity of these signatures will be established using tandem mass spectrometry, database mining and authentic standards. Metabolites of interest would eventually be quantified using in-house GC-MS or triple-quadrupole mass spectrometer as deemed appropriate. Finally receive operating characteristics (ROC) analysis will be performed to evaluate the sensitivity and specificity of metabolic signatures in classifying diabetic and nondiabetic kidney disease patients.

Challenges and alternatives: 1) One of the major issues in identification of metabolite of interest is unavailability of authentic standards. In such case, synthesis of putative target compounds would

be carried out followed by comparative fragmentation analysis with and without spiking, 2) In case, there are too many candidates for an unknown signature, chromatographic separation followed by FTIR and/or NMR-based analysis would be attempted for identification.

Peptidomics of urine

Apart from peptides, urine also contains proteins that would compete and interfere with ionization of peptides in mass spectrometer, which might lead to decrease in analytical sensitivity. In order to remove proteins and enrich peptides, ultrafiltration followed by solid-phase extraction (SPE) will be performed. Urine samples will be thawed and centrifuged to remove any particulate matter. Supernatant will be added to solution containing urea and SDS (21) in washed ultrafiltration unit (MW cut-off 5 or 10 kDa) and centrifuged at 4°C. Concentrated solution will be cleaned up either by Zip-tip (Merck Millipore) and spotted directly on MALDI plate or by C18 desalting columns (such as, Sep-Pak, Waters Corp.) and vacuum-dried. Vacuum dried peptide mix will be reconstituted using aqueous acetonitrile containing TFA and spotted on MALDI plate. α-Cyano-4-hydroxy-cinnamic acid (CHCA) will be used as matrix and peptide composition will be analyzed on a MALDI TOF/TOF 5800 (Sciex) platform in reflectron mode. Fragmentation pattern of signals of interest will be examined in TOF/TOF mode and putative identification of the peptide will be carried out using MASCOT. Raw data will be aligned, deconvoluted and binnedto identify difference in peptide composition of the urine samples from DKD and NDKD patients.

Challenges and alternatives: 1) The increase in peptide abundance may cause competitive ionization and ion suppression leading to decrease in sensitivity. In such case, LC-MALDI or LC-ESI approach will be adopted would be used to separate analytes prior to mass spectrometric analysis. 2) The lack of reproducibility of MALDI might end up making it an inferior choice for quantitative analysis. In such case, MALDI with iTRAQ labelling and/or ESI-triple quad (label-free) platform would be used for quantitation of peptides of interest.

***Analysis of robustness of signatures*:** Samples will be collected at different time points of the day with or without empty stomach with varying amount of water consumption prior to sample collection. Samples will be stored at room temperature and/or 4°C and/or -20°C and/or -80°C for varying period of time prior to analysis with or without flash-freezing. They will also be stored with or without protease inhibitor. They will be exposed to multiple freeze-thaw cycles prior to analysis. The effect of these varying sample collection, storage and handling conditions on putative biomarkers will be examined by directly measuring the level of metabolite and peptides using mass spectrometry. Finally, ROC analysis will be performed to assess the effect of these variations on the ability of selected metabolites and peptides in differential diagnosis of DKD and NDKD.

Output of the project:

The present proposal aims to identify multi-panel protein and metabolite maker that would expect to differentiate between DKD and NDKD, present biochemical and clinical investigations are limited to this aspect. Presently available “gold test” is biopsy. Facilities for taking kidney biopsy are not available commonly, expensive and has high risk for the patients. Treatment and

management of NDKD is different from that of DKD. Thus identification of noninvasive markers in urine sample is expected to fill up the gap, Clinicians and patients will be benefited, if noninvasive marker could be identified. In addition, metabolites and peptides identified in urine may provide mechanistic insight into DKD and NDKD.

References:

1. Darshi M, Espen BV, Sharma K. Am J Nephrol 2016;44:92–103
2. Wetterstein HI, Weiss RH Organogenesis 9:1, 11–18
3. Xu EY, et al Chem Res Toxicol 2008; 21:1548-61
4. Boudonck KJ Toxicol Pathol 2009; 37:280-92
5. Nkuipou-Kenfack, E. et al PLoS ONE 9(5): e96955.
6. Kimura, T et al Sci Rep 2015; 6:26138
7. Ganti, S. Et al Cancer Res 2012; 72:3471-9
8. Ganti, S. Et al Int J Cancer 2012; 130:2791-800
9. Men, L et al RSC Adv., 2017, 7: 16494
10. Sharma K, et al J Am Soc Nephrol. 2013; 24:1901–1912
11. Van der Kloet FM, et al Metabolomics (2012) 8:109–119
12. Nolph KD, Ann Intern Med. 1977;86(1):93-97.
13. Clark WR, Winchester JF. Adv Ren Replace Ther. 2003 Oct;10(4):270-8.
14. Mullen W, et al Curr Opin Nephrol Hypertens. 2011 Nov;20(6):654-61
15. Mischak H et al, Nature Reviews Nephrology 2015, 11: 221–232
16. Pena MJ, Mischak H, Heerspink HJL. Diabetologia. 2016; 59: 1819–1831.
17. Debigaré R, Price SR. Am J Physiol Renal Physiol. 2003 Jul;285(1):F1-8.
18. Rajan V, Mitch WE. Biochim Biophys Acta. 2008 Dec;1782(12):795-9
19. Good DM, et al, Mol Cell Proteomics. 2010;9:2434–2437.
20. Betz BB et al, Kidney Int. 2016 May;89(5):1125-35.
21. Klein, J. et al. Kidney Int. 2016 Nov;90(5):1045-1055
22. Manna SK, et al J Proteome Res. 2010 Aug 6;9(8):4176-88 2010
23. Manna, SK et al Gastroenterology 2014 May;146(5):1313-24
24. Singh, A. et al J Clin Invest. 2013;123(7):2921-2934

Budget

(Figures are in Rupees in Lakhs)

Items	First year	Second year	Total

Biopsy for 100 cases @Rs 5000 per sample)	2.5	2.5	5.0
Other clinical tests including imaging (@ Rs3000/ per sample)	1.5	1.5	3.0
Solvent and chemicals, GC and LC derivatization reagents, Stable isotope labeled standards, Gas cylinders, gas purifiers, GC and LC columns, etc for metabolomics	5.5	5.5	11.0
Chemicals and consumables for peptidomics (Low MW cut-off spin columns, Zip-tips, SPE columns and accessories, etc)	3.3	3.2	6.0
Consumables (glassware, plasticware, gloves, etc)	1.0	1.0	2.0
Multivariate analysis softwares (SIMCA-P+)	1.4	-	1.4
Contingency	0.5	0.5	1.0
	15.7	14.2	29.9

Justification for consumables

(a) Proposed budget for biopsy and other clinical evaluation is necessary for assays required for clinical diagnosis confirming the clinical diagnosis by biopsy. It is proposed that 100 patients (50 samples in each year) will be recruited on the basis of clinical diagnosis and confirmed by biopsy. For biopsy and related expenditure (@Rs5000/ per sample) and several other imaging and biochemical test (@ Rs 3000/ per sample) will be necessary.

(b) For metabolomic and peptidomic studies, various solvents, fine chemicals, GC and LC derivatization reagents, stable isotope labeled standards, Gas cylinders (for mass spectrometry), gas purifiers, GC and LC columns, solid-phase extraction columns will be necessary. In addition, a specific software namely SIMCA-P+ (multivariate analysis software) is essential for analyzing the huge data generated during this study and to determine the signature.